University of Alberta

Novel Insights into the Role of O^6-Methylguanine-DNA Methyltransferase in Glioblastoma Angiogenesis, Invasion, and Proliferation

By

Manik Chahal

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DEDICATION

To my parents, for their unconditional love and support, and for teaching me to face every challenge with determination and a smile. I owe everything I am and everything I will be to them.

ABSTRACT

Glioblastoma multiforme (GBM) is characterized by aberrant angiogenesis, rapid proliferation, and widespread invasion through the brain parenchyma. Thus, the prognosis for these tumors remains extremely poor despite aggressive multimodal treatment including chemotherapy with the alkylating agent temozolomide (TMZ). The expression of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) further contributes to treatment resistance by repairing tumoral alkylation damage induced by TMZ, highlighting the requirement for alternative therapeutic options. Though *MGMT* promoter methylation is a prognostic indicator of response to TMZ, it has also been correlated with increased rate of mutation in GBM and phenotypic alterations in other tumors. Therefore, we aimed to identify whether MGMT may play a greater role in GBM pathology and therapeutic response beyond mediation of alkylating chemotherapy resistance.

We determined through gene expression profiling that MGMT expression in GBM cells induced genetic alterations in several functional pathways. Of particular interest in GBM pathology, MGMT overexpression elicited a switch of the angiogenic balance towards an anti-angiogenic profile, as determined by altered expression of vascular endothelial growth factor and its receptors. Furthermore, we established an inverse relationship between MGMT expression and invasion using MGMT overexpression and knockdown models and panels of established and patient-derived primary GBM cell lines. Importantly, our *in vitro* data were validated in primary GBM tumor biopsy samples showing that *MGMT*

promoter methylation (i.e. *MGMT* gene silencing) was significantly associated with increased immunohistochemical expression of SPARC, a well-known marker of GBM invasion. Concurrently, in isogenic models we found that MGMT expression positively correlated with increased proliferation, consistent with the well-established theory that invasion and proliferation are mutually exclusive in GBM. Of great importance, the clinical relevance of our investigations was further demonstrated by our striking finding that decreased MGMT expression was associated with increased invasiveness following treatment with angiogenic inhibitors.

Our study therefore provides new insight into the aggressiveness of GBM by highlighting potentially novel roles for the DNA repair protein MGMT. Moreover, our data suggest the potential utilization of MGMT as a biomarker for response to angiogenic inhibitors, which constitutes an important step in developing more effective therapies for patients suffering from this devastating disease.

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LIST OF ABBREVIATIONS

Abbreviations	Full name
5-aza	5-aza-2'-deoxycitidine
^{60}CO	cobalt-60
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
AQUA	automated quantitative analysis
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCNU	bis-chloroethylnitrosourea
BMDC	bone marrow-derived cell
BTIC	brain tumor initiating cell
c-KIT	stem cell growth factor receptor
C/EBPβ	CCAAT-enhancer-binding protein β
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CGI	CpG island
CML	chronic myeloid leukemia
CNS	central nervous system
CSF1-R	colony stimulating factor-1 receptor
CSF3	colony stimulating factor-3
DAPI	4', 6'-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DIC	differential interference contrast
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNMT3a	DNA methyltransferase 3a
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EORTC	European Organization for Research and Treatment of Cancer
EV	empty vector
FAK	focal adhesion kinase
FCS	fetal calf serum
FDA	Food and Drug Administration
FFPE	fresh-frozen paraffin-embedded
FGF	fibroblast growth factor
Flt-1	FMS-like tyrosine kinase 1 (vascular endothelial growth factor receptor-1)
FLT3	FMS-like tyrosine kinase 3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GBM	glioblastoma multiforme
GEP	gene expression profiling
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GIST	gastrointestinal stromal tumor
GO	Gene Ontology
Gy	Gray
H/E	hematoxylin/eosin
HIF-1α	hypoxia inducible factor-1α
IDH-1	isocitrate dehydrogenase-1
i.p	intraperitoneal
IGF-1	insulin-like growth factor-1
IgG_1	immunoglobulin 1
IHC	immunohistochemistry
Kb	kilobase
KDR/Flk-1	kinase insert domain receptor/fetal liver kinase-1/vascular endothelial growth factor receptor-2
KEGG	Kyoto Encyclopedia of Genes and Genomes
KPS	Karnofsky Performance Scale
LCM	laser capture microdissection
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MBD2	methyl-CpG binding domain protein 2
MDM2 or 4	murine double minute 2 or 4
MeCP2	methyl-CpG binding protein 2
MGMT	O^6-methylguanine-DNA methyltransferase
MMP	matrix metalloproteinase
MMR	mismatch repair
MRI	magnetic resonance imaging
MSP	methylation-specific polymerase chain reaction
MT1-MMP	membrane type-1 matrix metalloproteinase
MTIC	5-(3-methyltriazin-1-yl) imidazole-4-carboxamide
NCAM	neural cell adhesion molecule
NCIC	National Cancer Institute of Canada
NF-1	neurofibromatosis-1
O6BG	O^6-methylguanine
O6MG	O^6-benzylguanine
P/S	penicillin/streptomycin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PE	phycoerythrin
PI3K	phosphatidylinositol-3-kinase
PLGF	placental growth factor

PLL	poly-L-lysine
PTEN	phosphatase and tensin homolog
PTK2	protein tyrosine kinase 2
QRT-PCR	quantitative real-time polymerase chain reaction
RB	retinoblastoma
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
RT	radiation therapy
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SF	sorafenib tosylate
shRNA	small hairpin RNA
siRNA	small interfering RMA
SPARC	secreted protein acidic and rich in cysteine
STAT3	signal transducer and activator of transcription 3
SU	sunitinib malate, SU11248
TCGA	The Cancer Genome Atlas
TGF	transforming growth factor
TIMP-1	tissue inhibitor of metalloproteinase-1
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TMZ	temozolomide
TRITC	tetramethylrhodamine-5-(and 6)-isothiocyanate
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WBRT	whole brain radiation therapy
WHO	World Health Organization
Wt	wild-type
XTT	sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

Chapter 1: Introduction

1.1. GLIOBLASTOMA MULTIFORME: PATHOLOGY, HALLMARKS, AND TREATMENT

The brain plays a central role in all aspects of bodily function. Thus, the dramatic functional disturbances that can arise due to minimal alterations of the neural circuitry or cytoarchitecture account for the severity of many brain tumors. Gliomas are the most common primary brain tumor, accounting for approximately 40% of all central nervous system (CNS) malignancies (DeAngelis 2001). Among these, astrocytomas are the most prevalent and are classified by the World Health Organization (WHO) based on histological features of the tumor, from grade I to grade IV. Glioblastoma multiforme (GBM) (a grade IV astrocytoma) is the most frequent and most aggressive form of malignant brain tumor in adults, with an annual incidence of about 3 in every 100 000 (Louis, Ohgaki et al. 2007).

Though GBM has a relatively low incidence compared to other cancers, it is associated with disproportionately high morbidity and mortality due to its highly aggressive phenotype, characterized by rapid tumor growth and infiltration. Despite optimal treatment, the median survival for patients with GBM is only 12-15 months. Patients with gliomas present with a variety of symptoms, including seizures, focal neurological deficits, personality changes, memory loss, physical weakness, and headaches (Wen and Kesari 2008). Although the reported incidence of many asymptomatic benign CNS tumors is increasing due to advances in diagnostic imaging, the rapid and aggressive growth of GBMs usually precludes their incidental discovery (Adamson, Kanu et al. 2009). It is not uncommon for patients with GBM to experience the onset of symptoms abruptly

since GBM can occasionally remain asymptomatic until it reaches an enormous size, which can occur in a significantly short period of time (Iacob and Dinca 2009).

Often, the types of symptoms presented reflect the location of the tumor in the brain. GBM is preferentially located supratentorially in the subcortical white matter of the cerebral hemispheres, with frequency highest in the frontal lobe, followed by temporal, parietal, and finally occipital lobe (Larjavaara, Mantyla et al. 2007; Zada, Bond et al. 2011). Occasionally GBM can present in the corpus callosum displaying the characteristic "butterfly appearance" of bihemispheric involvement (Agrawal 2009). Conversely, infratentorial GBM of the cerebellum or brain stem is rare in adults and can be difficult to diagnose because of non-specific symptoms and radiological features (Stark, Maslehaty et al. 2010).

Currently, no underlying cause has been identified for GBM. While the incidence of gliomas is reported to be rising (Hess, Broglio et al. 2004; Dobes, Khurana et al. 2011), predisposing factors to GBM remain poorly understood because of its multifaceted nature. Interestingly, the incidence is fairly consistent worldwide, making GBM a considerable public health issue.

1.1.1. GBM heterogeneity: pathology and origin

The extraordinary heterogeneity of GBM is evident at every pathological level: from macroscopic, to microscopic, and to molecular. With magnetic resonance imaging (MRI) GBM classically appears as a heterogeneous hypointense lesion indicative of necrosis, surrounded by an enhancing ring of

highly proliferative and angiogenic viable tumor. Beyond the enhancing borders is a nonenhancing region of infiltrative edema that represents both the extensive peritumoral vasogenic edema frequently associated with GBM and migrating tumor cells that can disseminate 1-3 cm from the apparently well-circumscribed mass (Cha 2006).

Pathologically, malignant gliomas (grade III and IV astrocytomas) are classified based on the presence of increased cellularity, nuclear atypia, and mitotic activity, while GBM is further marked by microvascular proliferation and/or necrosis (Wen and Kesari 2008). GBM additionally possesses strikingly diverse features histologically. The term "multiforme" was coined to reflect the variegated appearance of the cells which ranged from small, intense blue cells to huge, multinucleated giant cells that could coexist within the same tumor (Scherer 1940). Furthermore, malignant gliomas typically contain both neoplastic and stromal cells which additionally contribute to histologic heterogeneity and variable outcome (Wen and Kesari 2008).

This heterogeneous population of cells exhibits various tumorigenic potentials, highlighting the requirement for understanding the cellular origin of GBM. Historically, gliomas were thought to arise from transformed astrocytes given their tendency to express high levels of an astrocyte-specific marker glial fibrillary acidic protein (GFAP) (McComb and Burger 1985). However, not all GBM tumor cells express GFAP. More recent evidence suggests that the GBM cell of origin may be a less differentiated cell type such as a multipotent glial progenitor cell or a stem cell-like brain tumor-initiating cell (BTIC)

(Siebzehnrubl, Reynolds et al. 2011). BTICs possess neural stem-like characteristics such as the ability to self-renew, extensively proliferate, and to generate new tumors that faithfully reproduce the heterogenic phenotype of the initial tumor from which it was derived (i.e. differentiate) (Galli, Binda et al. 2004; Singh, Hawkins et al. 2004; Vescovi, Galli et al. 2006). Accounting for tumor heterogeneity, a recent study by Chen et al. (2010) demonstrated a hierarchical organization of BTICs where subpopulations of clonal, long-term proliferating cells were shown to represent different stages of differentiation and give rise to tumors with different histopathological and molecular features (Chen, Nishimura et al. 2010).

1.1.2. GBM heterogeneity: subclassification and molecular biology

Histological grading accurately reflects the varied cellular appearance of GBM, but it does not reflect its diverse genetic characteristics. Currently GBM is separated into two main subtypes with discrete clinical presentations: primary GBMs which arise *de novo* and typically occur in patients over 50 years of age, and secondary GBMs that transform from a pre-existing lower-grade astrocytoma. The latter primarily occur in younger patients, and account for only 10% of all GBM cases (DeAngelis 2001). These two clinical subtypes possess notable molecular differences and outline the classical view of GBM genetics (Figure 1.1).

In GBM, aberrant receptor tyrosine kinase (RTK) signaling pathways may be the most often cited genetic defects. Amplification of epidermal growth factor

receptor (EGFR) is the most common of these genetic alterations, and is seen almost exclusively in primary GBM (Frederick, Wang et al. 2000). EGFR amplification is often associated with structural alterations of the gene, and approximately half of the tumors with EGFR amplification express a variant known as EGFRvIII characterized by a truncated extracellular domain with ligand-independent constitutive activity (Pelloski, Ballman et al. 2007). Autophosphorylated EGFRvIII continuously triggers downstream mitogenic signaling pathways to promote growth, proliferation, migration, and neovascularization (Huang, Nagane et al. 1997), and can mediate resistance to radiation and chemotherapy (Montano, Cenci et al. 2011). Platelet derived growth factor receptor (PDGFR), a key regulator of glial development (Kesari and Stiles 2006), is similar to EGFR and is frequently amplified in secondary GBM. Importantly, both the receptor and PDGF ligand are frequently overexpressed and can create an autocrine loop stimulating tumor proliferation (Furnari, Fenton et al. 2007) and angiogenesis (Dunn, Heese et al. 2000).

Although loss of heterozygosity (LOH) of chromosome 10q is common in both primary and secondary GBM, the mutation or deletion of phosphatase and tensin homolog (*PTEN*), a tumor suppressor gene located on chromosome 10q, is most commonly attributable to primary GBM (Ohgaki, Dessen et al. 2004). *PTEN* negatively regulates the phosphatidylinositol-3-kinase (PI3K)-Akt pathway, which is associated with many biological functions in cancer. Consequently, inactivation of *PTEN* leads to decreased inhibition of the PI3K-Akt pathway

resulting in increased cell survival, proliferation, and invasion (Cully, You et al. 2006).

While *PTEN* deletion is a defining characteristic of primary GBM, the hallmark of secondary GBM is mutation in the *TP53* gene (Ohgaki, Dessen et al. 2004). *TP53* encodes p53, an important transcription factor that regulates a large number of genes in response to a variety of cellular insults including oncogene activation and DNA damage. Additionally, p53 is known to play a role in the cell cycle, cell differentiation, and neovascularization (Bogler, Huang et al. 1995). *TP53* mutations most often result in the inability of p53 to regulate transcription (i.e. loss-of-function), thereby promoting enhanced cellular proliferation (described in section 1.1.5) and genetic instability, which can lead to neoplastic transformation of a lower grade astrocytoma to GBM (Nozaki, Tada et al. 1999). There is also increasing evidence that mutant forms of p53 can gain new oncogenic properties (Brosh and Rotter 2009), such as the ability to promote cell invasion by enhancing integrin and EGFR trafficking (Muller, Caswell et al. 2009).

Though these alterations are characteristic of primary or secondary GBM, none of them are specific enough to distinguish between the two GBM subtypes. Furthermore, both primary and secondary GBMs are morphologically indistinguishable and prognosis does not seem to differ after adjustment for age (Wen and Kesari 2008). Thus there exists a requirement for improved subclassification of GBM into different prognostic groups. This may be accomplished by gene expression-based molecular profiling.

A comprehensive genomic analysis conducted by Parsons et al. (2008) elucidated multiple genes not previously known to be altered in GBM, and most notably, identified mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene, which is involved in energy metabolism. This study revealed *IDH1* mutation to be a potentially more specific marker for secondary GBM and a subpopulation of primary GBM that seem to arise from clinically silent low-grade tumors (Parsons, Jones et al. 2008). Moreover, subsequent studies have shown that tumors with *IDH1* mutations had clinical and pathological features that identified them as a discrete group with less aggressive disease and significantly longer survival than those with wild-type *IDH1* (Sanson, Marie et al. 2009; Lai, Kharbanda et al. 2011). Based on another integrated genomic analysis using data from the Cancer Genome Atlas (TCGA), Veerhak et al. (2010) identified 4 molecular subclasses of GBM: neural, proneural, classical, and mesenchymal, which respond differently to aggressive therapy and have distinct similarities to different neural lineages (Verhaak, Hoadley et al. 2010). The actual utility of this study and similar genomic studies is yet to be determined, as numerous groups have attempted to delineate prognostic subgroups of GBM but have thus far lacked consistency and reproducibility (Marko, Quackenbush et al. 2011). Nonetheless, regardless of the subclassification of GBM, to some degree they all exhibit the hallmarks of heightened angiogenesis, invasion, and proliferation.

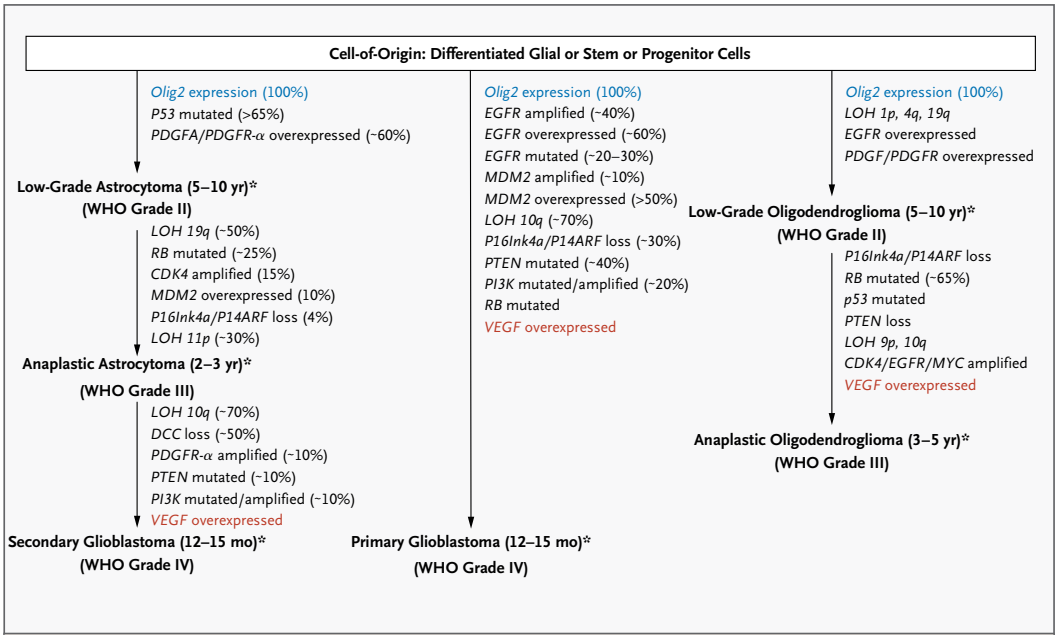

Figure 1.1. Classical view of genetic alterations in the development of malignant gliomas. Frequency of genetic and chromosomal alterations involved in the development of the 3 main types of malignant gliomas (primary and secondary glioblastomas and anaplastic oligodendroglioma) is shown with median lengths of survival (asterisks). (Adapted from (Wen and Kesari 2008) *New England Journal of Medicine*)

1.1.3. Angiogenesis in GBM

Because of the brain's high oxygen dependence and sensitivity to increased intracranial pressure, there are unique consequences related to tumor growth in this location. Normal brain vasculature is highly specialized. It is composed of endothelial cells, pericytes, and astrocytes, which form and maintain the blood-brain barrier (BBB) that selectively restricts the exchange of molecules between the intracerebral and extracerebral circulatory systems (Deeken and Loscher 2007). When tumors grow beyond a 1-2 mm diameter within the brain parenchyma, the BBB becomes compromised both structurally and functionally (Wolburg, Noell et al. 2012). For instance, compression of blood vessels by cancer cells can increase resistance to blood flow and impair blood supply to vital brain structures, resulting in a spatially and temporally heterogeneous network. Disruption of the BBB can also increase the likelihood of spontaneous hemorrhage. Furthermore, vasogenic edema is a common side effect of BBB damage and is a significant cause of morbidity associated with GBM. Patients suffering from vasogenic edema often require chronic corticosteroid use which can lead to additional steroid-related toxicities such as osteoporosis, weight gain, insomnia, infection, and psychiatric effects (Gerstner and Batchelor 2012).

GBMs develop their vascular supply through multiple mechanisms. Recent investigations have suggested that vasculogenesis, i.e. the *de novo* formation of blood vessels via recruitment of endothelial progenitor cells or bone marrow-derived hematopoietic cells, mediates GBM vascularization and revascularization at recurrence (Lyden, Hattori et al. 2001; Greenfield, Cobb et al.

2010; Kioi, Vogel et al. 2010). However, a majority of studies suggest that the primary etiology of glioma neovascularization is angiogenesis, more specifically "sprouting angiogenesis", which is defined as the induction and creation of new blood vessels by proliferation of endothelial cells from pre-existing adjacent vessels. Judah Folkman hypothesized in 1971 that solid tumors would be unable to grow beyond a microscopic size of 1-2 mm^3 without continuous recruitment of new blood vessels for nutrient delivery and waste removal (Folkman 1971), and gliomas appear to obey this central tenet of angiogenesis. Regardless of the mechanism, vascular changes accompany the progression of gliomas and are included in the criteria for glioma grading, with GBM being the most vascularized among astrocytic tumors (Brem 1976; Louis, Ohgaki et al. 2007).

The appearance of the vasculature also differs between grades, with low-grade gliomas possessing vasculature that resembles that of normal brain, while high-grade gliomas show prominent microvascular proliferation and higher vascular density compared to normal brain (Plate and Risau 1995). These tumor vessels are structurally and functionally abnormal. Excessive endothelial cells along with abnormal perivascular cells contribute to the formation of tortuous, distended, and saccular blood vessels that are poorly organized and hyperpermeable (Jain, di Tomaso et al. 2007). As a result of vascular hyperpermeability, tumor vessels are unable to maintain gradients between vascular and interstitial pressures. The increased interstitial fluid in the tumor causes fluid to leak out of the tumor into the surrounding tissue, raising the cerebrospinal fluid pressure until it ultimately becomes equal to the interstitial

fluid pressure, thereby contributing to vasogenic edema and consequently morbidity (Gerstner and Batchelor 2012).

At the histological level, GBM neovascularization is associated with the formation of complex and bizarre vascular formations, i.e. glomeruloid structures, vascular garlands, and vascular clusters (Wesseling, van der Laak et al. 1998). These structures are composed of several closely associated capillaries and endothelial sprouts surrounded by variably thickened basement membrane (Rojiani and Dorovini-Zis 1996). The endothelial cells lining the vessels show evidence of hyperplasia, increased numbers of Weibel-Palade bodies (which store von Willebrand factor and P-selectin in endothelial cells), and focal distensions in the interendothelial junctions that restrict the diameter of the vessel (Coomber, Stewart et al. 1987). GBM also exhibits evidence of "classic" angiogenesis characterized by delicate, evenly distributed capillary-like microvascular sprouting. In a study by Birner et al. (2003) the combination of low bizarre vasculature formations and prominent classic vascular pattern was found to be an independent factor for longer survival, suggesting that vascular patterns in GBM may influence clinical outcome (Birner, Piribauer et al. 2003).

The angiogenic switch, occurring early in tumorigenesis, is induced by the overexpression of pro-angiogenic factors in response to genetic/mutational signals and environmental signals such as hypoxia through a mechanism primarily mediated by the transcription factor hypoxia inducible factor-1α (*HIF-1α*) (Kaur, Khwaja et al. 2005). Several pro-angiogenic factors and their cognate receptors have been elucidated as important contributors to GBM pathology, such as

platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factors (TGFs) (Dunn, Heese et al. 2000), angiostatin, endostatin, thrombospondin (Anderson, McFarland et al. 2008) and others.

Arguably however, the most important contributor to angiogenesis is vascular endothelial growth factor (VEGF). Though there are numerous members of the VEGF ligand family [VEGF-A, -B, -C, -D, -E, and placental growth factor (PLGF)] (Byrne, Bouchier-Hayes et al. 2005), VEGF-A (commonly referred to as simply VEGF) is the most potent stimulator of angiogenesis and is thought to be the principal driver of GBM neovascularization (Plate, Breier et al. 1992). Compared to normal astrocytes that do not synthesize VEGF, glioma cells abundantly produce VEGF mRNA and protein (Fischer, Gagner et al. 2005), and in general, high-grade gliomas produce more VEGF than lower grade astrocytomas (Chaudhry, O'Donovan et al. 2001). VEGF binds to its cognate receptors belonging to the RTK family, primarily VEGFR-1 (fms-like tyrosine kinase Flt-1) and VEGFR-2 (kinase insert domain receptor KDR/ Flk-1), which is the principal mediator of several physiological and pathological effects of VEGF on endothelial cells. Ligand binding to VEGFR-2 induces receptor dimerization which leads to auto-phosphorylation at tyrosine residues and phosphorylation of further intracellular proteins such as PI3K and mitogen-activated protein kinases (MAPKs), which promote survival, proliferation, migration, and vascular permeability (Figure 1.2) (Ferrara 2004; Olsson, Dimberg et al. 2006; Jain, di Tomaso et al. 2007). Neoplastic VEGF action was traditionally thought to be

attributable to a paracrine mechanism: glioma cells devoid of cell surface VEGFRs secrete high levels of VEGF, which bind to numerous VEGF receptors located on endothelial cells that produce little or no VEGF themselves (Kerbel 2008). Recent studies conversely suggest that VEGF may also fuel autocrine pro-survival processes in GBM, as VEGFR-2 has been identified on GBM cells (Knizetova, Ehrmann et al. 2008), and specifically in BTICs expressing the glioma stem cell marker CD133 (Hamerlik, Lathia et al. 2012). VEGFR-1, which has weaker tyrosine kinase activity compared to VEGFR2, but 10x higher affinity for VEGF (Park, Chen et al. 1994) has also been identified on GBM cells (Mentlein, Forstreuter et al. 2004). Though its role in GBM is still poorly understood, many authors regard VEGFR-1 as a "decoy receptor" not involved in VEGF signal transduction but in regulation of VEGF activity by binding without cellular effects (Seetharam, Gotoh et al. 1995). Soluble VEGFR-1 (sVEGFR-1) produced by alternative splicing sequesters the VEGF ligand and can also antagonize VEGF-VEGFR-2 signaling (Kendall and Thomas 1993; Hornig and Weich 1999). Thus the ability of VEGF to elicit its pro-angiogenic effects depends on the relative concentration of these receptors in the GBM tumor and its vasculature.

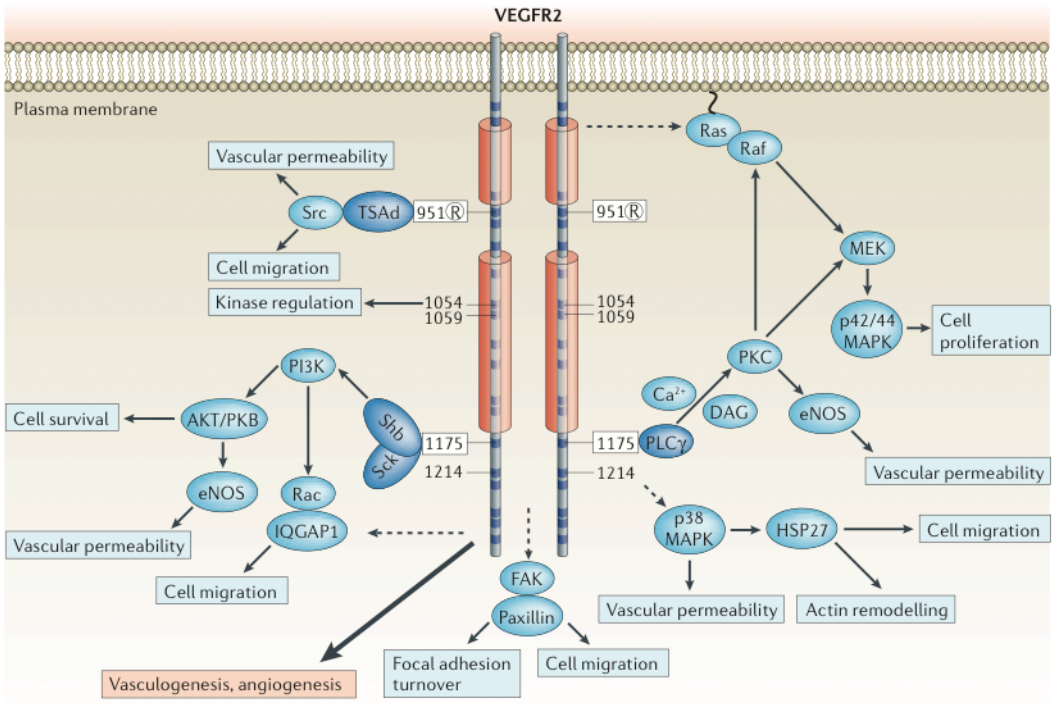

Figure 1.2. VEGFR-2 signal transduction in endothelial cells. Intracellular domain of dimerized and activated VEGFR-2 is shown with tyrosine (dark blue squares in the receptor) phosphorylation sites (indicated by numbers). The circled R indicates that use of the phosphorylation site is regulated dependent on the angiogenic state of the receptor. Pale blue boxes indicate specific biological responses based on signaling cascades. The final biological outcome that is coupled to the receptor is vasculogenesis and angiogenesis (indicated in the pink box). (Adapted from (Olsson, Dimberg et al. 2006), *Nature Reviews. Molecular Biology*)

1.1.4. GBM invasion

In addition to being highly angiogenic, GBM is a rapidly invasive tumor. Both angiogenesis and invasion are requisites for GBM growth and dissemination, and these two hallmarks display inherent similarities (Vajkoczy, Goldbrunner et al. 1999; Eccles 2004). GBM cells typically invade the brain as single cells along myelinated fiber tracts (Giese, Kluwe et al. 1996), along the basement membrane of blood vessels, within perivascular spaces, and in the subependyma surrounding the lateral ventricles (Giese and Westphal 1996; Bellail, Hunter et al. 2004). The high affinity for myelin fiber tracts enables the formation of satellite lesions several centimeters away from the initial tumor site, and lesions as far as the contralateral hemisphere are not uncommon (Gaspar, Fisher et al. 1992; Van Meter, Dumur et al. 2006).

Consequently GBM invariably recurs even following extensive surgical resection including hemispherectomy (Matsukado, Maccarty et al. 1961; Furnari, Fenton et al. 2007). This suggests that diffusely disseminating, individual invasive cells may possess properties of BTICs (discussed in section 1.1.1). Although a number of groups have suggested that BTICs are found exclusively within the tumor core rather than the invasive edge (Mangiola, Lama et al. 2007; Glas, Rath et al. 2010; Pistollato, Abbadi et al. 2010), several lines of evidence support the hypothesis that invasive cells have tumor initiating capabilities.

Increased invasiveness was recently correlated with stem cell properties in various types of cancer including GBM (Wakimoto, Kesari et al. 2009), and the expression of the glioma stem cell marker CD133 (Singh, Clarke et al. 2004) was

shown to be significantly higher in GBM patients with diffusely disseminated lesions compared to those with more localized tumors (Sato, Sakurada et al. 2010). Furthermore, invading GBM cells are known to express mesenchymal markers (Carro, Lim et al. 2010), and cancer cells that acquire a mesenchymal phenotype are also known to acquire stem cell properties (Mani, Guo et al. 2008). In a xenograft model of GBM, invasive cells also showed a more pronounced phenotype consistent with stem cell characteristics compared to cells acquired from the tumor core, such as the ability to form neurospheres *in vitro*, the expression of the glioma stem cell marker nestin, and increased tumorigenicity (Molina, Hayashi et al. 2010). Chicoine and Silbergeld (1995) found that when C6 rat glioma cells that had invaded into the contralateral hemisphere were reestablished in culture and implanted into naïve rats, they formed large tumors, thus confirming the retained stem cell capabilities of these cells (Chicoine and Silbergeld 1995). Additionally, in GBM patients with recurrent tumors, the second tumor was shown to have similar genetic profile to the primary tumor even after a 2-year period following its removal (van Nifterik, Elkhuizen et al. 2006).

The widely accepted process of GBM invasion requires 4 concerted steps: cell detachment from the primary tumor site, attachment to the extracellular matrix (ECM), remodeling of the ECM, and finally morphological alterations resulting in migration (Figure 1.3) (Nakada, Nakada et al. 2007). First, to detach from the nascent tumor mass, invasive cells must deregulate adhesion molecules involved in cell-cell interactions. For instance, neural cell adhesion molecule (NCAM), which is expressed on developing neurons and mediates homophilic

binding, is reported to have decreased expression in invasive high-grade gliomas (Sasaki, Yoshida et al. 1998). Similarly, all gliomas regardless of grade appear to lack expression of E-cadherin, a molecule that contributes to the formation of homotypic adherens junctions between cells (Nakada, Nakada et al. 2007). Conversely, though its precise role in GBM is inconclusive, N-cadherin is purported to be upregulated in invasive GBM cells based on evidence that in breast cancer cells its overexpression promoted migration (Hazan, Phillips et al. 2000), and based on the observation that an N-cadherin inhibitor was found to suppress invasion of U87 glioma cells (Takino, Nakada et al. 2003).

Next, the detached GBM cell must carefully coordinate between adhesion to and disengagement from the surrounding ECM. The composition of the ECM itself is an important mediator of the ability of a cell to migrate and invade (Chintala, Gokaslan et al. 1996). The majority of ECM proteins in the brain (such as fibronectin, laminin, collagen, vitronectin, and tenascin) are localized to the perivascular space where GBM invasion preferentially occurs (Demuth and Berens 2004). Additionally, the rigidity of ECM is directly correlated to GBM cell invasion, suggesting that ECM influences GBM invasion both biochemically and biophysically (Ulrich, de Juan Pardo et al. 2009). The molecules most recognized for their importance in ECM adhesion are from the integrin family of receptors. Integrins are transmembrane receptor proteins that bind to ligands in the ECM at localized focal adhesion sites. Upon ligand binding they form heterodimers between α and β subunits, subsequently initiating the assembly of intracellular proteins [notably among them focal adhesion kinase (FAK)] that

create a structural and molecular link between integrins and actin fibers of the cell cytoskeleton (Hood and Cheresh 2002; Natarajan, Hecker et al. 2003; D'Abaco and Kaye 2007). The β_1 integrin subunit is key because of its ability to partner many α units in the perivascular region, and because of its well-characterized correlation with glioma invasive behavior (Friedlander, Zagzag et al. 1996; Paulus, Baur et al. 1996). Heterodimers involving the α_v subunit (particularly $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$) have also been shown to be important for progression to high-grade gliomas (Gladson 1996), migration (Friedlander, Zagzag et al. 1996), and angiogenesis (Friedlander, Brooks et al. 1995). As a result, cilengitide, which targets $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins to inhibit angiogenesis and invasion, is being investigated as a potential therapy for GBM (Stupp, Hegi et al. 2010).

Invading cells must also express proteases at their leading edges capable of degrading the ECM ahead and allowing space through which they, and following cells, can migrate. Strong correlations have been reported between GBM invasiveness and expression of matrix metalloproteinases (MMPs), which hydrolyze a wide spectrum of extracellular proteins, activate signal transduction cascades promoting motility, and cleave and activate other growth factors sequestered in the ECM (Rao 2003). In particular, the gelatinases MMP-9 and MMP-2 are frequently expressed invasion-mediating factors in GBM (Nakagawa, Kubota et al. 1996; Choe, Park et al. 2002; Lakka, Gondi et al. 2004; Sarkar and Yong 2009). Furthermore, expression of the membrane type-1 MMP (MT1-MMP) that activates MMP-2 and acts as an ECM degradation enzyme is also highly expressed in GBM (Yamamoto, Mohanam et al. 1996; Chernov, Sounni et

al. 2009). Notably, the expression of all 3 MMPs increase with glioma progression (Forsyth, Wong et al. 1999).

Migration through newly remodeled ECM necessitates an alteration in morphology. Glioma cells specifically appear to use a mesenchymal mode of migration, typified by a polarized extension of leading edge membrane processes (such as pseudopodia, lamellipodia, filopodia, and invadopodia) in the direction of migration (Zhong, Paul et al. 2010). These extensions contain polymerized actin which is organized into short, branched filaments at the leading edge and longer tension-bearing filaments in the cytoplasm known as stress fibers (Friedl and Wolf 2003). To drive mesenchymal cell movement, actin filaments must undergo coordinated assembly and disassembly at the leading and trailing edge, respectively. This is accomplished by the cooperative activity of the small GTPases Cdc42, Rac, and RhoA, which was found to positively correlate with astrocytoma malignancy (Nobes and Hall 1999; Salhia, Rutten et al. 2005; Yan, Chour et al. 2006). Additionally, cell locomotion requires contractile force, and when connected to focal adhesion sites actin stress fibers contract due to integration with myosin II (Cramer 1999). As a result, invasive GBM cells migrate in a characteristic "stick-slip" pattern where the cell extends a prominent leading cytoplasmic process followed by an abrupt detachment of the trailing edge and a burst of forward movement in the cell body (Ulrich, de Juan Pardo et al. 2009).

Although these molecules play an important role in the mechanism of invasion, no single molecule has been identified as a marker for invasive GBM.

Several invasion gene candidates have been described, including insulin-like-growth-factor-binding proteins, angiopoietin 2, ephrins, secreted protein acidic and rich in cysteine (SPARC, described in section 1.1.5), and of great interest, YKL-40 (CHI3L1), a secreted glycoprotein (Nakada, Nakada et al. 2007). Aberrant expression of YKL-40 is associated with a variety of human diseases. In the context of GBM, YKL-40 was first identified as a potential serum marker that correlates with astrocytoma grade (Tanwar, Gilbert et al. 2002), which was validated by an independent study in 2005 (Nutt, Betensky et al. 2005). Since then its expression has been associated with increased radioresistance, poor survival, increased VEGF expression, and progression of GBM (Pelloski, Mahajan et al. 2005; Francescone, Scully et al. 2011). YKL-40 has also been explicitly linked to GBM invasion in a subtractive hybridization screen intended to elucidate genes expressed in invasive GBM compared to pilocytic astrocytoma (Colin, Baeza et al. 2006), and has been effectively used as a mesenchymal marker of invasive GBM *in vivo* (Carro, Lim et al. 2010).

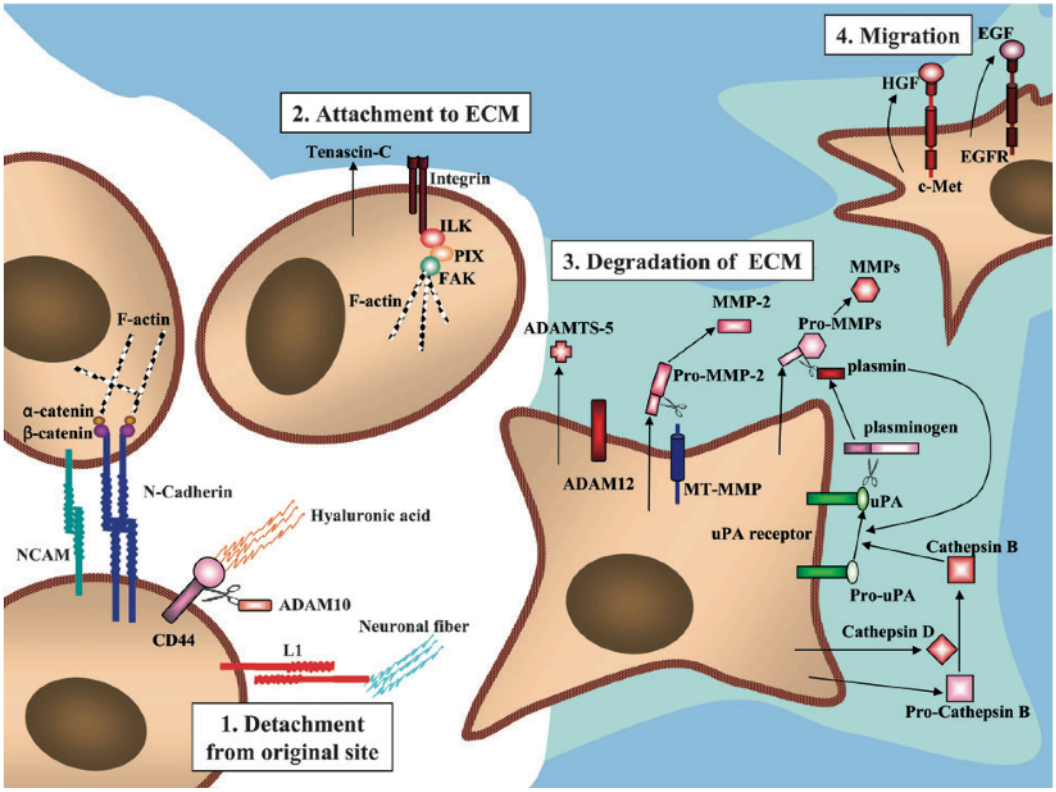

Figure 1.3. Putative mechanism of glioma invasion. Potential mediators of each step are depicted. (Adapted from (Nakada, Nakada et al. 2007), *Cellular and Molecular Life Sciences*)

1.1.5. Proliferation of GBM and the migration/proliferation dichotomy

While the upregulation of invasive mechanisms accounts for the diffuse dissemination of the GBM tumor throughout the brain, deregulated proliferative mechanisms account for its rapid growth. At the histological level proliferative activity is usually quite prominent in GBM, with detectable mitoses present in nearly every case. Mitotic counts are therefore an important factor in malignancy grading of astrocytic tumors (Louis, Ohgaki et al. 2007). Regional proliferative heterogeneity has been demonstrated in GBM, with highest mitotic activity extending along the periphery of the solid tumor mass (i.e. the enhancing region when observed by MRI) (Coons and Johnson 1993; Dalrymple, Parisi et al. 1994). Detection of mitotic activity is most commonly accomplished by immunohistochemistry (IHC) using antibodies against Ki-67 and proliferating cell nuclear antigen (PCNA). The concentration of PCNA/cyclin, a nuclear auxiliary protein of polymerase δ (involved in DNA replication), is highest when cells are in the G_1/S phase (Bravo and Macdonald-Bravo 1987). On the other hand, Ki-67 is an antigen expressed in all phases of the cell cycle except for G_0, and is therefore considered a more sensitive and specific marker of GBM proliferation than PCNA (Gerdes, Lemke et al. 1984; Kordek, Biernat et al. 1996; Torp and Granli 2001). Accordingly, the highly proliferative phenotype of GBM is attributed to loss of cell cycle control and additional positive regulation of cell cycle progression mediated by increased growth factor receptor signaling.

The two key regulatory pathways controlling progression through the cell cycle are the p53-mediated pathway and the $p16^{INK4a}$/cyclin-dependent kinase

(CDK)-4/retinoblastoma (RB) pathway. In normal cells p53 is activated following DNA damage to guard against inappropriate progression through cell cycle checkpoints. p53 accomplishes this by inducing transcription of genes encoding p21, which subsequently binds and inhibits CDK2. The activity of CDK2 is important for G_1-S progression (Sherr and Roberts 1999). Additionally, in damaged cells the $p14^{ARF}$ tumor suppressor prevents degradation of p53 by mouse double minute 2 (MDM2) ubiquitin ligase, ensuring that p53 is present to inhibit aberrant progression through the cell cycle (Brooks and Gu 2004). Similarly, the RB pathway also controls the G_1-S checkpoint. When the CDK4/6-cyclin D1 complex phosphorylates RB, it induces the release of the E2F transcription factor that activates genes involved in the G_1-S transition (Sellers and Kaelin 1997). The $p16^{INK4a}$ tumor suppressor blocks the phosphorylation of RB by binding to CDK4, and consequently prevents unnecessary progression through the G_1 checkpoint (Serrano, Hannon et al. 1993) (Figure 1.4). These two pathways are commonly disturbed in GBM by several mechanisms. The tumor suppressor proteins $p14^{ARF}$ and $p16^{INK4a}$ are located on the same genetic locus, and primary GBMs are commonly associated with abnormalities in this locus thus preventing their appropriate expression. Both primary and secondary GBM can be additionally marked by mutated p53 (described in section 1.1.2), loss of pRB function, CDK4 amplification, or MDM2 amplification, thereby resulting in uncontrolled and aggressive tumor proliferation (Lang, Miller et al. 1994; Biernat, Tohma et al. 1997; Soni, King et al. 2005).

Increased autocrine and paracrine signaling resulting from overexpression of growth factors and their receptors further amplify GBM proliferation. As described in section 1.1.2, EGF and PDGF, as well their cognate receptors EGFR and PDGFR are overexpressed in GBM. Amplification of TGF-α and insulin-like growth factor-1 (IGF-1) and their cognate receptors is also associated with GBM and is thought to boost tumor growth (Nister, Libermann et al. 1988; Trojan, Cloix et al. 2007). Activation of these receptors leads to propagation of downstream signaling via two primary intracellular protein cascades, the Ras/Raf/MAPK(ERK) pathway and the PI3K/AKT pathway, which consequently induce transcription of genes involved in the cell cycle. Additional deregulation can occur within the pathways through aberrant expression of any one of the mediators, or inactivation of their repressors PTEN (which inhibits AKT activation, described in section 1.1.2) or neurofibromatosis-1 (NF-1, which inhibits the ERK pathway) (Roberts and Der 2007; Gottfried, Viskochil et al. 2010; Lino and Merlo 2011).

Importantly, besides mediating proliferation, these intracellular pathways influence a diverse array of vital cellular functions including migration. While these pathways can be stimulated by mitogenic growth factors, cytokines and ECM components can activate both ERK and AKT pathways simultaneously to trigger motility and invasion (Roberts and Der 2007; Lino and Merlo 2011). Moreover, inhibitory cross-talk can occur between the two pathways, and since the ERK pathway is considered to be primarily associated with proliferation while the AKT pathway is frequently linked to invasion (Zimmermann and Moelling

1999; Sunayama, Matsuda et al. 2010), the convergence of these signaling cascades suggests that invasion and proliferation may be coordinated behaviors. It is believed there is an inherent inverse correlation between proliferation and invasion in GBM, a phenomenon termed the migration/proliferation dichotomy (Giese, Loo et al. 1996; Fedotov and Iomin 2007). Theoretically, a heightened commitment to either proliferation or motility results in the diminution of the other process, as cellular machinery can only be occupied with one of these processes at a time (Berens and Giese 1999; Hatzikirou, Basanta et al. 2012). The seminal study by Giese et al. (1996) showed that when a glioma cell population was plated on a migration-permissive substrate *in vitro*, the front of migrating cells showed reduced proliferation compared to the immobile cells in the center of the colony (Giese, Loo et al. 1996). Further supporting this concept, it has been found that the proliferative index of cells located at the edge of a GBM tumor mass is significantly higher than of invasive cells extending into the brain parenchyma (Dalrymple, Parisi et al. 1994), and that infiltrating glioma cells do not appear to enter mitosis (Silbergeld and Chicoine 1997). A cDNA microarray analysis additionally revealed that glioma motility is associated with reduced transcription of genes involved in proliferation (Mariani, Beaudry et al. 2001).

Due to the complexity of mitogenic and motogenic signaling pathways, little is known about how cells make the decision to either proliferate or invade. However, specific genes have been implicated in the dichotomous regulation of these two processes. For example, since PDGFRs trigger both motility and mitosis, the type and concentration of the activating ligand can influence whether

cells migrate or divide. Specifically, when NIH3T3 fibroblasts were treated with exogenous PDGF, concentrations higher than 5 ng/mL were required to induce cell proliferation, while cell migration responses started at 1 ng/mL and were negligible at higher concentrations (De Donatis, Comito et al. 2008). EGFR signaling has also been implicated in this dichotomy, and the decision to migrate or proliferate appears to depend on binding and activity of the G-protein Gαi3 to the intracellular domain of EGFR (Cho and Kehrl 2007; Ghosh, Garcia-Marcos et al. 2008; Ghosh, Beas et al. 2010). Specifically in GBM, increased expression of secreted protein acidic and rich in cysteine (SPARC), a glycoprotein that inhibits cell adhesion to ECM (Sage 1997), has been correlated with increased invasion and delayed cell growth (Schultz, Lemke et al. 2002; Seno, Harada et al. 2009). It accomplishes this regulation by mediating integrin and growth factor receptor signaling (Raines, Lane et al. 1992; Thomas, Alam et al. 2010), modulating the expression of MMPs (McClung, Thomas et al. 2007), and arresting cells in the G_0/G_1 phase of the cell cycle (Rempel, Golembieski et al. 2001). Additionally, microRNA-451 has been characterized as a conditional switch controlling glioma invasion and proliferation (Godlewski, Bronisz et al. 2010). High miR-451 expression promotes cell proliferation but decreases migration in an energy-dependent manner (Godlewski, Nowicki et al. 2010). Recently, overexpression of the ephrinB2 receptor has also been shown to inhibit GBM neurosphere proliferation while stimulating cell migration and invasion through a FAK-mediated mechanism (Wang, Rath et al. 2012).

The migration/proliferation dichotomy in GBM has major implications for therapy, as invasive cells with low proliferative capacity would be relatively resistant to conventional cytotoxic treatments directed against proliferating cells (Roos and Kaina 2006). Accordingly, migratory cells have been shown to be more resistant to apoptosis caused by cytotoxic insult (Mariani, Beaudry et al. 2001; Giese, Bjerkvig et al. 2003).

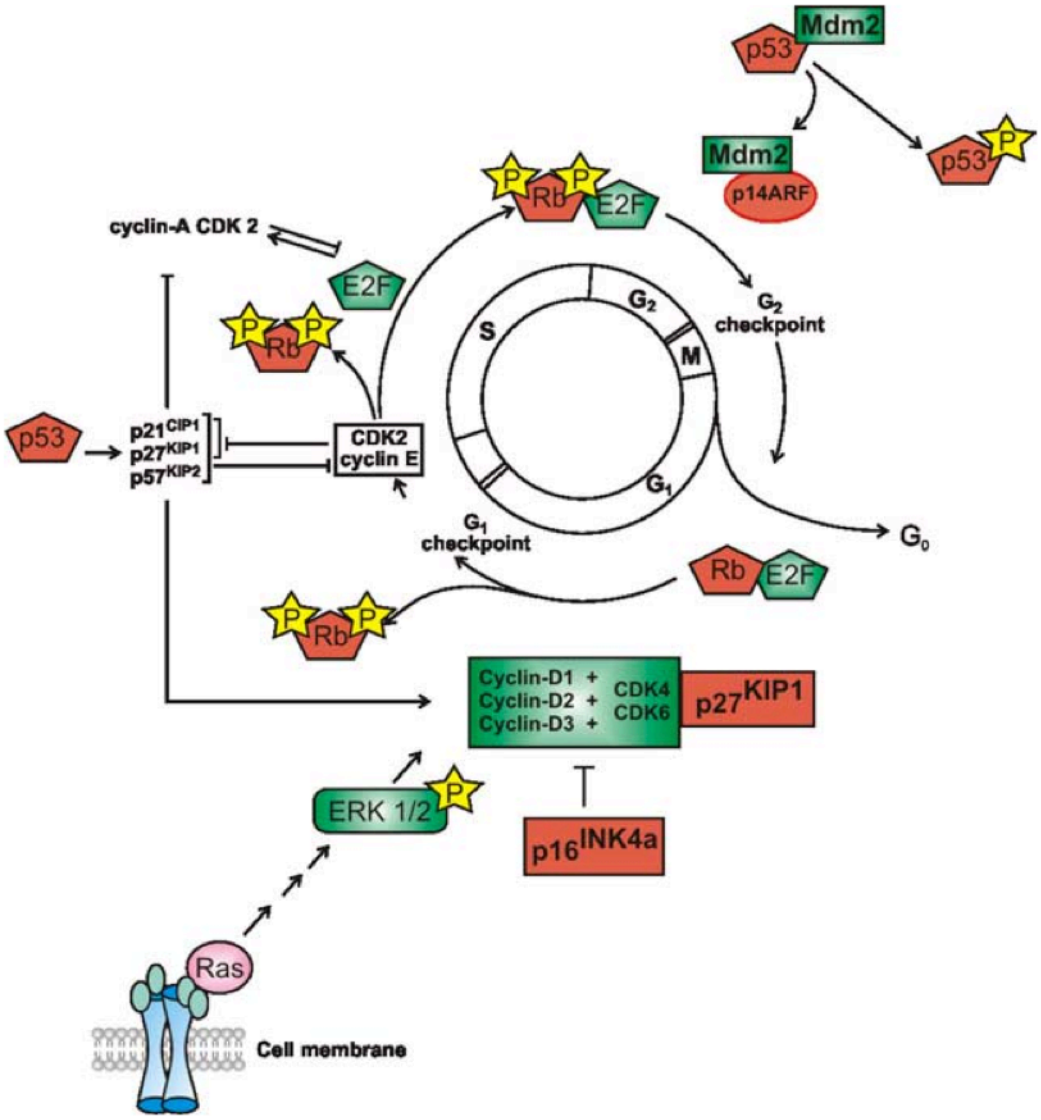

Figure 1.4. Regulatory control of the cell cycle. Molecules depicted in red are known or putative tumor suppressor proteins, while those in green are known or potential oncogenes promoting growth. Phosphorylation is indicated as yellow stars. (Adapted from (Soni, King et al. 2005), *Journal of Clinical Neuroscience*)

1.1.6. Treatment of GBM

The standard therapy for newly diagnosed GBM involves maximal surgical resection when feasible and adjuvant radiation and chemotherapy depending on age and performance as measured by the Karnofsky Performance Scale (KPS). Although GBM cannot be completely eliminated surgically because of its highly infiltrative nature (as described in section 1.1.4), surgical debulking can reduce the symptoms from mass effect such as neurological deficits or raised intracranial pressure, and can provide tissue for histologic diagnosis and molecular studies (Wen and Kesari 2008; Adamson, Kanu et al. 2009). Advances in neuroimaging have increased the extent of surgical resection to greater than 98% depending on location and confers a significant increase in survival over subtotal resection (Lacroix, Abi-Said et al. 2001; Stummer, Reulen et al. 2008). However, gross total resection only appears to improve survival in the short term, since 2-year disease-free survival is unchanged in patients who receive maximal resection compared to biopsy alone (Stewart 2002).

The addition of external-beam radiation therapy (RT) to surgery has consistently demonstrated efficacy in treating GBM, based on the ability of ionizing radiation to nonspecifically target rapidly dividing cells and damage DNA. Several randomized clinical trials have tested the efficacy of whole brain RT (WBRT) as a method of targeting the tumor mass as well as infiltrative GBM cells throughout the brain. Although recurrences distal to the initial tumor site were significantly lower in patients receiving WBRT compared to localized RT, there was no significant benefit in overall survival (Shapiro, Green et al. 1989).

Localized external-beam RT is therefore the routine treatment for newly diagnosed GBM since it both limits the risk of radiation damage to healthy brain tissue and maintains an average survival of 7-12 months compared to 3-6 month survival with surgery alone (Walker, Alexander et al. 1978; Laperriere, Zuraw et al. 2002). RT is administered in a standard regimen of 30 fractions over a course of 6-7 weeks for a total dose of 60 Gy (or an abbreviated course of 40 Gy over 15 fractions in older patients) to a target volume defined as a 2-3 cm ring of tissue beyond the contrast enhancing rim of tumor seen on the preoperative MRI scan (Laperriere, Zuraw et al. 2002). Notably neither dose escalation, alternate fractionation schedules (particularly hyperfractionation), brachytherapy, nor stereotactic radiosurgery have demonstrated a significant survival advantage (Wen and Kesari 2008). Therefore the onus lies on chemotherapeutics to provide additional benefit for GBM patients.

Numerous chemotherapies have been tested in GBM, and alkylating agents appear to hold the most promise. Alkylating agents target highly proliferating cells by initiating apoptosis in a replication dependent manner. DNA chloroethylating nitrosoureas, particularly bis-chloroethylnitrosourea (BCNU, carmustine), have historically been the most commonly used chemotherapeutics for GBM (Walker, Green et al. 1980). However, BCNU can cause considerable side effects, and upon further analysis, has been found to have only very modest effects on patient survival (Brandes, Tosoni et al. 2004).

Despite four decades of research with BCNU, a large randomized phase III clinical trial coordinated by the European Organization for the Research and

Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) in 2005 established temozolomide (TMZ), a methylating agent with a more acceptable side effect profile compared to BCNU, as the new standard of care for newly diagnosed GBM. Comparing RT alone to RT with concomitant and adjuvant TMZ, treatment including TMZ was found to increase median survival from 12.1 months to 14.6 months. Additionally, the survival rate at two years was 26.5% for the RT plus TMZ group compared to 10.4% for the RT alone group (Stupp, Mason et al. 2005). This significant increase in survival was validated upon 5-year analysis, as overall survival was 9.8% for the group treated with TMZ compared to 1.9% for the group treated with RT alone (Stupp, Hegi et al. 2009).

TMZ is a lipophilic pro-drug capable of crossing the blood brain barrier, and at physiological pH it spontaneously hydrolyzes to the active intermediate 5-(3-methyltriazin-1-yl) imidazole-4-carboxamide (MTIC). MTIC in turn gives rise to a highly reactive methyldiazonium species that interacts with and methylates nucleophilic DNA bases (Tentori and Graziani 2009) (Figure 1.5). TMZ-mediated DNA methylation can occur at several different base sites and generates a wide spectrum of methyl adducts represented mainly by N^7-methylguanine (70%) and N^3-methyladenine (9%) (Newlands, Stevens et al. 1997). However, its antitumor activity is mainly attributed to the O^6-methylguanine (O6MG) lesion that accounts for only 5% of methylation induced by TMZ. The toxicity induced by unrepaired O6MG derives from the erroneous pairing of the methylated base with thymine instead of cytosine during DNA synthesis (Marchesi, Turriziani et al. 2007)

(described in more detail in section 1.2). This mismatch eventually initiates apoptosis via the death receptor (Fas-dependent) pathway or mitochondrial pathway depending on the p53 status of the cell (Roos and Kaina 2006).

Despite the utility of TMZ in GBM therapy, median survival times are still remarkably low for patients. As described in section 1.1.5, this may be a result of a diminished proliferative capacity of highly invasive cells. Additionally, several mechanisms exist that may underlie resistance to TMZ, such as the prevalence of BTICs (Beier, Schulz et al. 2011), disturbances of the mismatch repair system (Mirzoeva, Kawaguchi et al. 2006; Yip, Miao et al. 2009), p53 status (Bocangel, Finkelstein et al. 2002), or the amplification of EGFR polymorphisms (Puyo, Le Morvan et al. 2008; Montano, Cenci et al. 2011). Interestingly, since EGFR can regulate DNA repair machinery and consequently the response to DNA damaging agents (Squatrito and Holland 2011), recent investigations have examined the efficacy of "combi-molecules" designed to possess mixed EGFR targeting as well as DNA methylating properties (Banerjee, Huang et al. 2011).

However, the ability of alkylating agents to induce lasting DNA damage primarily appears to rely on the activity of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) (Huang, Rachid et al. 2011). MGMT is regarded as the leading factor involved in cancer resistance to alkylating agent-based chemotherapy, and specifically is the best-described resistance factor in TMZ-treated glioma patients (Stupp, Hegi et al. 2009; van Nifterik, van den Berg et al. 2010).

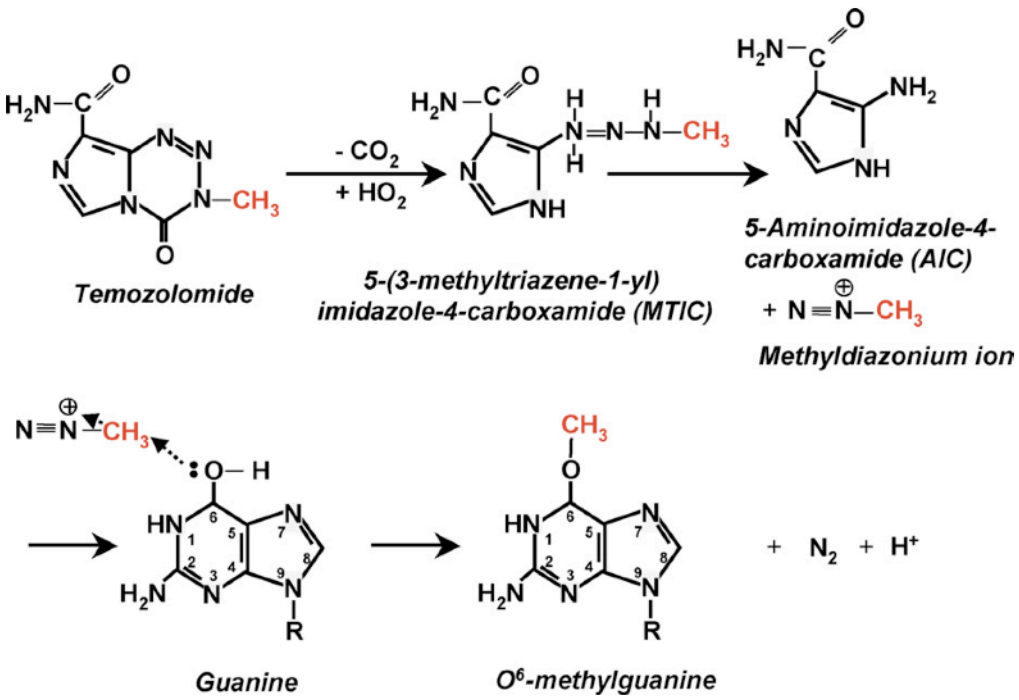

Figure 1.5. Mechanism of O6MG formation by temozolomide. (Adapted from (Kaina, Christmann et al. 2007), *DNA Repair*)

1.2. O^6-METHYLGUANINE-DNA METHYLTRANSFERASE: SIGNIFICANCE IN GBM

O^6-methylguanine-DNA methyltransferase (MGMT) is a well-characterized protein with one putative function: repair of DNA alkylation. MGMT is ubiquitously expressed in normal human tissues. However, expression levels vary considerably between individuals and between organs, with highest levels usually found in the liver and relatively low levels found in the brain (Gerson, Trey et al. 1986; Gerson 2004). Thus, MGMT protects both normal and tumor cells from the mutagenic effects of exogenous and endogenous alkylating agents by removing alkyl adducts primarily from the O^6 position of guanine through a process of direct reversal (Esteller, Garcia-Foncillas et al. 2000).

O^6-methylguanine (O6MG) lesions are highly mutagenic, and efficient repair is essential for cell integrity. If these lesions remain unrepaired, DNA polymerases stall at the aberrant base during DNA replication and incorrectly insert thymine opposite of O6MG instead of cytosine, the standard binding partner of guanine, resulting in a mutation on the daughter strand (Gerson 2004). The O6MG:T mismatch is then typically recognized by the mismatch repair (MMR) system that subsequently cleaves out the thymine residue along with a section of the daughter strand. However, because the O6MG residue remains in the parental DNA strand, the MMR complex repeatedly inserts the wrong nucleotide base opposite O6MG. This initiates a faulty repair cycle resulting in single-strand breaks and eventually double-strand breaks that block replication in the next cell cycle and lead to apoptosis (Karran and Bignami 1994; Kaina,

Christmann et al. 2007). Accordingly, MGMT is a vital repair protein that can minimize the damage induced by alkylating agents in healthy cells. However, MGMT, when functional in GBM cells, can significantly reduce the efficacy of chemotherapeutic agents such as TMZ that rely on the formation of DNA alkyl adducts.

The first observations indicating a potential predictive role of MGMT in glioma patients were made over 15 years ago, when it was found that patients with low MGMT protein levels showed greater benefit from treatment with BCNU compared to patients with high MGMT levels (Belanich, Pastor et al. 1996). Since then there has been significant preclinical and clinical evidence for a role of MGMT in dictating response to alkylating agents in GBM. In fact, an overwhelming majority of studies on MGMT have focused on this relationship. In a landmark companion study to the EORTC-NCIC trial by Stupp et al. (2005) advocating TMZ as standard treatment, Hegi and colleagues showed that added benefit of TMZ is only conferred to patients whose tumors display *MGMT* promoter methylation (which silences protein expression, described in section 1.2.2), with a 2-year survival rate of 46% compared to 14% for patients with unmethylated *MGMT* tumors (Hegi, Diserens et al. 2005). This difference was confirmed after five years when 14% of patients with *MGMT*-methylated tumors survived compared to 8% survival of patients with unmethylated tumors. Of note, 12 out of 92 patients with *MGMT* methylated tumors survived, while only 6 out of 114 patients with *MGMT* unmethylated tumors survived after 5 years in all treatment groups (Stupp, Hegi et al. 2009). Importantly, *MGMT* methylation

status is not only a powerful predictive marker of increased sensitivity to alkylating therapeutics, but is also considered to be the strongest prognostic factor for outcome in patients with newly diagnosed GBM (Criniere, Kaloshi et al. 2007; Weller, Stupp et al. 2010; Olson, Brastianos et al. 2011) and in elderly patients treated with concomitant and adjuvant TMZ (Brandes, Franceschi et al. 2009).

Currently the determination of *MGMT* promoter status is conducted whenever possible, as it may assist in the determination of prognosis. However, MGMT status is only assessed in a few cancer centers, and regardless, it does not influence the administration of TMZ to all GBM patients. Though the percentage of GBM patients possessing tumors with methylated *MGMT* promoters varies between reports, a variety of studies have indicated high MGMT tumor content in 60% or more of newly diagnosed GBM patients (Chamberlain 2010). Consequently, over half of all treated GBM patients derive no additional benefit from chemotherapy with TMZ.

1.2.1. MGMT structure and mechanism of action

The *MGMT* gene is located on chromosome 10 at the 10q26 position (which, as described in section 1.1.2 is commonly lost in GBM), and consists of 5 exons and 4 introns spanning greater than 300 kilobases (kb) (Natarajan, Vermeulen et al. 1992). The promoter region lacks the constitutive regulatory elements known as the TATA box and CAAT box, and contains a CpG island rich in repetitive GC sequences (Weller, Stupp et al. 2010). The region required for

maximal promoter activity consists of a minimal promoter, an enhancer region, and several transcription factor-binding sites (Sharma, Salehi et al. 2009).

Its encoded protein MGMT, found within both the nucleus and cytoplasm of the cell (Belanich, Randall et al. 1996), is 207 amino acids in length and is highly conserved phylogenetically (Bugni, Han et al. 2007). It is comprised of two domains, with residues involved in DNA binding, nucleotide flipping, and the active site pocket located at the C-terminal domain (Tubbs, Pegg et al. 2007). Within this domain the active site sequence proline-cysteine-histidine-arginine is also highly conserved. The function of the N-terminal domain is not completely understood, but it is thought to play a critical structural role in maintaining the C-terminal domain in an active configuration (Fang, Kanugula et al. 2005). The N-terminal domain also contains a bound zinc atom shown to increase the rate of repair (Rasimas, Kanugula et al. 2003).

The essential conserved function of MGMT is alkyl transfer (Gerson 2004). Although methyl groups at the O^6 position of guanine are its preferred substrate, MGMT is also able to remove more complex alkylations including O^6-ethylguanine, O^6-chloroethylguanine and, to a much lesser extent, O^4-methylthymine (Verbeek, Southgate et al. 2008). Alkyl group removal is accomplished without generating DNA breakage and without the aid of other enzymes or cofactors, followed by direct transfer of the adduct from the mutated base to a cysteine residue (cys-145) in the active site of the MGMT protein (Grafstrom, Pegg et al. 1984) (Figure 1.6). The covalent attachment of the alkyl adduct to cys-145 initiates an inactivating conformational change in MGMT

characterized by disruption of the hydrogen bonding network involving cys-145 and opening of the asparagine hinge that releases MGMT from the DNA (Xu-Welliver and Pegg 2002). This conformational change also increases the ability of the protein to serve as a substrate for ubiquitin ligases that tag MGMT for subsequent degradation by the 26S proteasome (Srivenugopal, Yuan et al. 1996). MGMT is therefore a suicide protein with 1:1 stoichiometry, in that one MGMT protein is required to repair one alkyl adduct. Consequently, the ability of a cell to withstand alkylating damage is directly related to the number of MGMT molecules it contains and the rate of *de novo* synthesis (Yarosh 1985; Esteller and Herman 2004).

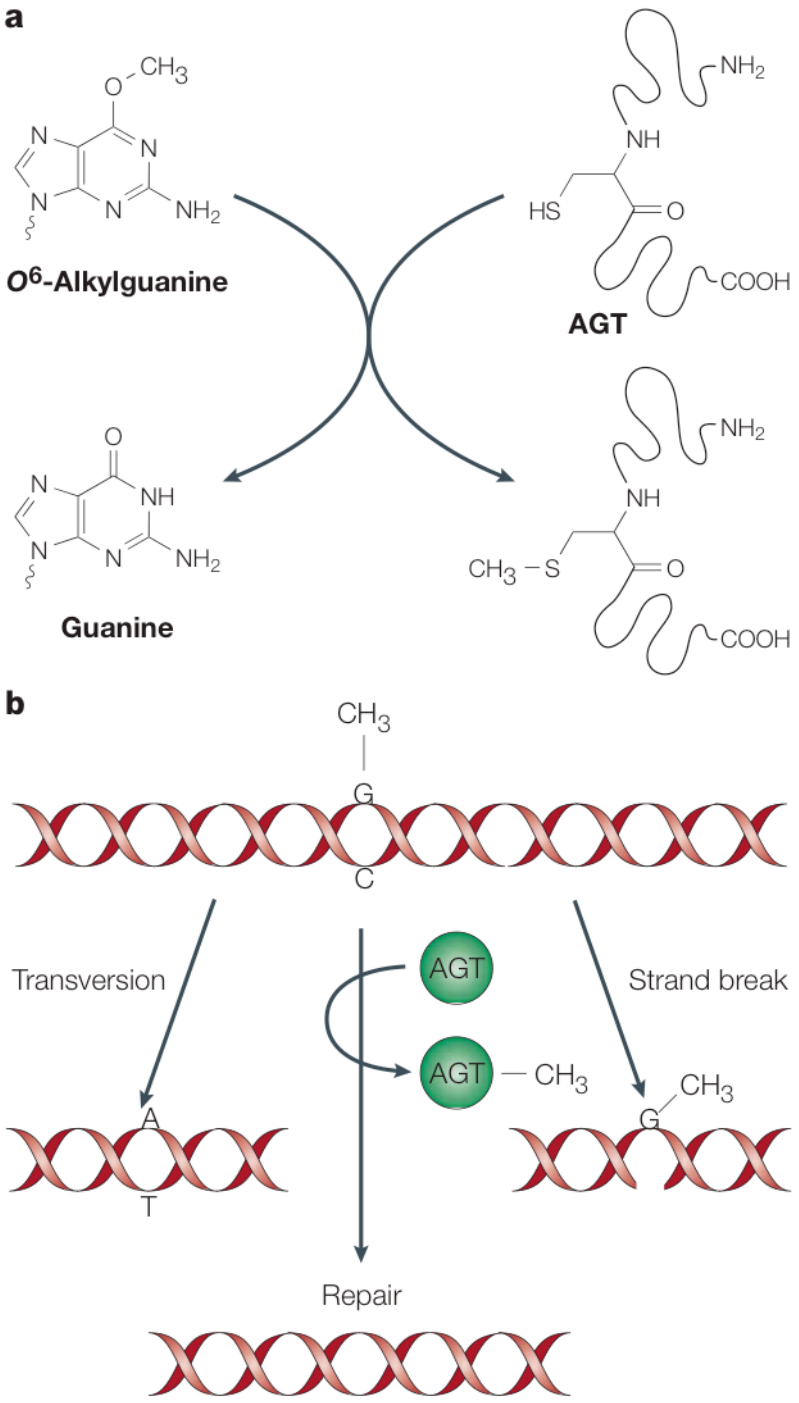

Figure 1.6. MGMT repair process. (A) MGMT scans double-stranded DNA for alkylations at the O^6 position of guanine and covalently transfers the adduct to a cysteine residue in its active site and restores the guanine residue to normal. **(B)** If repair of O6MG does not occur, a G→A transition mutation or strand break can result. (Adapted from (Gerson 2004), *Nature Reviews. Cancer*)

1.2.2. Regulation of MGMT expression in cancer

Though MGMT is a DNA repair protein with tumor suppressive function, several tumors including gliomas frequently exhibit higher MGMT expression than corresponding normal tissue (Citron, Graver et al. 1992; Silber, Blank et al. 1996). The fact that there are several transcription sites in the promoter region of MGMT explain why MGMT may be upregulated in some cancers, since it can be induced by glucocorticoids, cyclic AMP, and protein kinase C activators (Gerson 2004).

Regarding inactivation of MGMT in brain neoplasms, which as previously described predicts response to TMZ, hypermethylation of the CpG islands in the *MGMT* promoter, rather than mutation or deletion, is the primary mechanism of MGMT loss of function (Esteller, Hamilton et al. 1999). Two regions of the promoter that are prone to high levels of methylation have been identified, and methylation of the region comprising the enhancer element appears to be more critical for the loss of MGMT expression (Everhard, Tost et al. 2009). Upon methylation of *MGMT*, methyl-CpG binding proteins such as methyl-CpG binding protein 2 (MeCP2) and methyl-CpG binding domain protein 2 (MBD2) are recruited to the aberrantly methylated sequences, and subsequently instigate the formation of inactive chromatin in a "closed nucleosome" structure. This limits transcription factor binding and ultimately, MGMT expression (Nakagawachi, Soejima et al. 2003).

Though MGMT levels vary in different tissues, *MGMT* promoter methylation is only observed in tumor cells (Gerson 2004), suggesting factors

other than epigenetic silencing by methylation may alter MGMT expression in both normal and neoplastic cells. Notably, several reports have proposed that wild-type (wt) *TP53* gene may act as a negative regulator of MGMT expression by reducing its transcription in normal cell and tumor models including glioma (Harris, Remack et al. 1996; Grombacher, Eichhorn et al. 1998; Srivenugopal, Shou et al. 2001). Specifically, overexpression of p53 has been shown to have an inhibitory effect on both basal *MGMT* promoter activity and its activation by DNA damaging agents (Grombacher, Eichhorn et al. 1998), and was found to increase tumor cell line sensitivity to alkylating agents (Srivenugopal, Shou et al. 2001). Furthermore, a recent study suggested that wild-type p53 abrogates MGMT expression by sequestering the Sp1 transcription factor and preventing its binding to the cognate cis-elements in the *MGMT* promoter (Bocangel, Sengupta et al. 2009). Conversely, other studies have shown that p53 induces MGMT expression in glioma cells by directly binding to the *MGMT* promoter (Blough, Zlatescu et al. 2007), even though the presence of a p53-binding site in the promoter region is debated. The relationship between MGMT and p53 may also be reversed, as MGMT inactivity has been suggested to regulate mutations in p53 (Esteller, Risques et al. 2001; Wolf, Hu et al. 2001; Bello, Alonso et al. 2004). These discrepancies indicate that the proposed mechanisms by which MGMT is regulated require further evaluation.

1.2.3. Issues regarding MGMT expression in GBM

Though the importance of MGMT expression in GBM is undeniable, establishing the MGMT status of a GBM tumor is unfortunately not straightforward. Firstly, changes in the methylation status of MGMT can occur at tumor recurrence following treatment with RT and TMZ, and appear to be more frequent in patients with methylated tumors at first occurrence (Brandes, Franceschi et al. 2010; Christmann, Nagel et al. 2010; Jung, Jung et al. 2010). This may reflect a selection for MGMT-expressing [i.e. MGMT(+)] chemoresistant tumor cells, as MGMT-nonexpressing [i.e. MGMT(-)] cells may be eliminated with treatment. Selection of a subpopulation of cells by chemotherapy reveals another important facet contributing to the issue of determining MGMT status: that of intratumoral MGMT heterogeneity.

There is significant debate over whether MGMT status is homogeneous or heterogeneous throughout the GBM tumor. Hamilton et al. found that MGMT status was homogenous using methylation-specific polymerase chain reaction (MSP) analysis of promoter methylation in frozen tissue samples from different regions of the tumor (specifically, the non-enhancing inner region, the enhancing rim, and the area immediately adjacent to the enhancing rim) (Hamilton, Roldan et al. 2010). Similarly, other groups showed that MGMT status is homogeneous throughout a tumor as determined by promoter methylation status, promoter sequencing, and protein expression by IHC (Grasbon-Frodl, Kreth et al. 2007; Parkinson, Wheeler et al. 2008; Cao, Jung et al. 2009). Conversely, numerous studies indicate that MGMT status within a GBM tumor is heterogeneous. For

instance, differences in *MGMT* methylation were observed in 50% of patients in which samples were extracted from the same tumor at least 3 mm apart (Juillerat-Jeanneret, Bernasconi et al. 2008). Additionally, in a method similar to Hamilton et al. where samples were taken from 3 concentric layers of tumors obtained from treatment-naïve newly diagnosed GBM patients, Della Puppa et al. (2011) observed that while a majority of patients did not exhibit any difference in MGMT promoter methylation between layers, MGMT protein expression progressively decreased from inner to outer layer (Della Puppa, Persano et al. 2011). Consequently, these findings indicate that the MGMT status of a GBM patient may not only depend on the location of the biopsy, but may also depend on the method utilized to determine the status.

Indeed, there is ongoing debate regarding how MGMT status should be assessed. Currently, MGMT status of a tumor is determined by promoter methylation analysis, since methylation was identified as a predictive marker for prolonged progression-free survival and overall survival in TMZ treated patients (Hegi, Diserens et al. 2005; Stupp, Mason et al. 2005). However, as the study by Della Puppa et al. (2011) shows, methylation does not always correlate with expression. As described in section 1.2.2, although methylation is the primary mechanism of *MGMT* gene silencing, it is not the only method of MGMT protein expression control. If the activity of MGMT in a tumor is the key determinant for chemoresistance, it can be argued that clinicians should be cautious when evaluating a therapeutic strategy based on *MGMT* methylation alone.

Though there have been indications of significant statistical agreement between *MGMT* methylation and protein expression (Everhard, Tost et al. 2009), several studies have reported an absence of correlation (Cao, Jung et al. 2009; Brell, Ibanez et al. 2011). Furthermore, while lack of MGMT protein expression has been purported to better predict response to TMZ (van Nifterik, van den Berg et al. 2010), others claim that protein expression alone cannot predict response (Karayan-Tapon, Quillien et al. 2010). These discrepancies may be indicative of limitations in detection methods.

Although MSP is the most commonly used test to determine *MGMT* promoter methylation (Esteller, Hamilton et al. 1999) other quantitative and semi-quantitative methods with increased sensitivity are also commonly used (Karayan-Tapon, Quillien et al. 2010). However, highly sensitive methods may overestimate the percentage of tumors in which methylation reaches a biologically meaningful level, since silencing of the *MGMT* gene requires dense methylation. In fact, quantitative assays have revealed a subgroup of patients with intermediate methylation, representing a "gray zone" in test results that may account for the fact that some *MGMT*-methylated tumors seem to derive no benefit from TMZ (Weller, Stupp et al. 2010). On the other hand, IHC for the detection of MGMT protein expression is easier to use, less expensive, and faster than MSP (Brell, Ibanez et al. 2011). Nevertheless its utility as a method to determine MGMT status remains controversial because of i) high inter-observer variability, ii) lack of established cut-offs to define low versus high expression, and iii) the fact that MGMT is expressed by tumor blood vessels, astrocytes, macrophages, and

activated microglia in addition to GBM tumor cells, making it difficult to identify and score tumor cells expressing MGMT (Nakasu, Fukami et al. 2007; Preusser, Charles Janzer et al. 2008).

1.2.4. MGMT, a hypermutator phenotype, and a potential role beyond chemotherapy sensitivity

Regardless of the method of detection, it is clear that loss of MGMT in a tumor cell improves response to alkylating chemotherapy. However, emerging evidence suggests that decreased MGMT expression may also contribute to tumor progression by enabling the acquisition of several mutations. Approximately 10-30 O6MG lesions can occur in a single cell per day resulting from exogenous and endogenous methylating agents (De Bont and van Larebeke 2004). Accordingly, a comprehensive genomic characterization of GBM tumors highlighted an association between *MGMT* promoter methylation and a hypermutator phenotype encompassing mutations in several genes (Network 2008). *MGMT* promoter methylation has been associated with the accumulation of mutations in several cancers, which have thus far been largely attributed to a failure in repairing O6MG. As described in section 1.2.1, alkylation triggers an erroneous pairing between O6MG and thymine during DNA replication, which can effectively result in a G:C→A:T transition. Based on an accumulation of these mutations, reduced MGMT expression is believed to increase the malignant potential of biliary tract cancer (Koga, Kitajima et al. 2005). Notably, loss of MGMT activity has also been associated with G:C→A:T transitions in *K-ras* (Esteller, Garcia-Foncillas et

al. 2000; Kohya, Kitajima et al. 2003), *PIK3CA* (Nosho, Kawasaki et al. 2008), and *TP53* (Esteller, Risques et al. 2001; Wolf, Hu et al. 2001; Bello, Alonso et al. 2004) in several tumors.

In addition to promoting G:C→A:T transitions, the O6MG residue has been purported to influence gene expression if left unrepaired. For instance, O6MG can inhibit the binding of transcription factors to DNA regulatory regions (Bonfanti, Broggini et al. 1991), thereby resulting in abnormal gene expression. Similarly, a recent study by Burns et al. revealed that the accumulation of O6MG lesions in MGMT-depleted cells are mutagenic at the level of transcription and subsequently induce alterations in protein function (Burns, Dreij et al. 2010). Furthermore, the nature of the neighboring base influences the extent of alkylation of guanine residues (Briscoe and Cotter 1984), and GC rich regions appear to be the preferred site for alkylation (Mattes, Hartley et al. 1988). Since cytosine methylation is important in the downregulation of certain genes, the presence of O6MG in promoter CpG islands can consequently impede the methylation of adjacent cytosine residues (Hepburn, Margison et al. 1991), and thereby inhibit normal gene silencing.

Evidence also suggests there may be an association between MGMT and phenotypic alterations of several tumor types. For instance, Konduri et al. found the *in vitro* inhibition of MGMT in pancreatic cancer cells decreased the expression of cyclins and Ki-67 while increasing the expression of $p21^{cip1}$, thus resulting in lowered proliferation (Konduri, Ticku et al. 2009). Interestingly, MGMT expression is decreased in invasive Crooke's cell adenomas when

compared to MGMT expression in non-invasive ordinary-type adenomas of Cushing's disease (Takeshita, Inoshita et al. 2009). Additionally, in esophageal cancer cells exposed to an alkylating agent *in vitro*, *MGMT*-silenced cells had more aggressive motility and invasive abilities compared to MGMT-proficient cells (Su, Liu et al. 2011). *MGMT* promoter methylation has also been associated with lymph node invasion and tumor stage in patients with gastric carcinoma (Park, Han et al. 2001), and shortened overall and disease-free survival of patients with oral and pharyngeal cancer (Taioli, Ragin et al. 2009). Furthermore, elevated expression of MGMT protein in patient biopsy samples was correlated with a slower rate of malignant transformation of diffuse astrocytoma compared to tumors with lower MGMT expression (Nakasu, Fukami et al. 2007). Importantly, Brandes et al. (2009) observed that following treatment, GBM tumors with *MGMT* promoter methylation were likely to recur at more distal sites from the initial tumor RT field, suggesting increased invasive potential of MGMT(-) cells (Brandes, Tosoni et al. 2009). A study of 225 GBM patient biopsy specimens also revealed that *MGMT* methylation might predict better response to RT alone, since the 2-year survival rate of patients with methylated tumors was 30% compared to only 16% survival in patients with unmethylated tumors (Rivera, Pelloski et al. 2010).

1.2.5. MGMT and protein interactions

The phenotypic alterations observed in GBM depending on MGMT status may be related to the DNA repair function of MGMT. However, there remains a

possibility that functional and physical interactions of MGMT with binding partners can account for the correlations observed between MGMT expression and phenotypic differences. Interestingly, Teo et al. (2001) found that MGMT co-immunoprecipitates with the transcription integrator CREB-binding protein CBP/300. Following alkyl transfer to the internal cysteine residue of MGMT and prior to ubiquitin-mediated degradation, the modified MGMT protein was instead found to bind and inhibit estrogen receptor, thereby repressing cell proliferation (Teo, Oh et al. 2001). In addition, proteomic analysis by Niture et al. in colon cancer cells identified over 60 MGMT-interacting proteins with diverse functions including those involved in DNA replication and repair, cell cycle progression, and RNA processing and translation (Niture, Doneanu et al. 2005). Together these investigations suggest that MGMT may have additional functions besides DNA repair.

1.2.6. Targeting MGMT in GBM

Since tumoral expression of MGMT limits the efficacy of current standard therapy for GBM, strategies to overcome MGMT-mediated resistance have been and are currently being explored. Because MGMT repairs DNA in a stoichiometric, auto-inhibitory fashion, the most popular approach to depleting MGMT is by direct inhibition using low molecular pseudosubstrates of MGMT. These O^6-methylguanine analogues, most notably O^6-benzylguanine (O6BG), bind to the active site of MGMT and induce its degradation, thereby allowing

alkylating agents to conduct their antitumor activity by creating lasting lesions in DNA (Dolan, 1990).

O6BG is a more effective substrate than O6MG because benzyl groups enter more readily into bimolecular reactions with MGMT. As a result, the methyl lesion induced by alkylating agents remains on the guanine residue in DNA. The reaction between O6BG and MGMT is very rapid and potent, and the rate increases with increasing concentration of O6BG (Rabik, Njoku et al. 2006). Studies have shown that significant resynthesis of MGMT has occurred 18 hours after O6BG administration (Schold, Kokkinakis et al. 2004). Therefore, the preferential binding between O6BG and MGMT gives damaged DNA time to attempt to replicate, which consequently results in tumor cell death. Indeed, O6BG administration was shown to restore sensitivity to alkylating agents such as BCNU and TMZ in preclinical brain tumor models (Mitchell, Moschel et al. 1992; Bobola, Silber et al. 2005; Rabik, Njoku et al. 2006). However, in a phase II clinical trial, though O6BG was able to restore TMZ sensitivity in patients with TMZ-resistant grade III anaplastic astrocytoma, there was no significant restoration of TMZ sensitivity in patients with TMZ-resistant GBM (Quinn, Jiang et al. 2009).

The increased sensitivity to alkylating damage conferred by the administration of O6BG can have negative consequences. Alkylating agents are only marginally toxic in most healthy cells because they do not rapidly proliferate. Hematopoietic cells however, are characterized by rapid proliferation, and as a result myelosuppression, or the decrease in production of blood cells in bone

marrow, is a principle toxicity associated with administration of alkylation agents (Verbeek, Southgate et al. 2008). Furthermore, low expression of MGMT in hematopoietic cells also likely contributes to alkylating agent-induced myelotoxicity (Gerson, Trey et al. 1986). As a result, because systemic administration of MGMT inhibitors such as O6BG causes depletion of MGMT in all tissues of the body, myelotoxicity can substantially increase, and dose reduction of alkylating drugs is often necessary to limit toxic side effects of treatment (Koch, Hundsberger et al. 2007). For example, in a clinical study combining O6BG and BCNU, the maximum tolerated dose of BCNU when combined with an MGMT-inhibitory dose of O6BG was approximately 3-fold lower than the maximum tolerated dose of BCNU alone (Schilsky, Dolan et al. 2000). Thus, because of the lack of alkylating agent selectivity for malignant tissue versus bone marrow, and the lack of selective MGMT depletion by MGMT inhibitors, there has been no demonstrated improvement in the therapeutic index for alkylating agents when used in conjunction with MGMT inhibitors (Helleday, Petermann et al. 2008).

MGMT imposes a clear resistance to standard therapy and has thus far been difficult to target therapeutically. Therefore alternative therapeutic options are required to improve outcome for both MGMT(+) and MGMT(-) GBM patients.

1.3. ANTI-ANGIOGENIC THERAPY: A NEW HOPE?

For as long as the concept of angiogenesis has been used to describe growth of solid tumors, the concept of anti-angiogenic therapy has been proposed as a potential treatment for cancer (Folkman 1972). Because of the predominance of angiogenesis in GBM (described in section 1.1.3) and the dismal prognosis of GBM patients despite current optimal therapy (described in section 1.1.6), the use of angiogenic inhibitors in GBM is a promising therapeutic strategy.

The first efforts to develop angiogenesis inhibitors occurred in the 1970s and 1980s by Judah Folkman and colleagues (Folkman 2007). Since then, the majority of angiogenic inhibitors under development have targeted the VEGF pathway (Sathornsumetee and Rich 2007), which is a critical mediator of angiogenesis and is commonly aberrantly regulated in GBM. By antagonizing the interactions between VEGF and its receptors (at either the ligand or receptor level), these agents were originally proposed to impede tumor growth by preventing neovascularization and pruning away existing vasculature, which would lead to deprivation of oxygen and nutrients resulting in tumor death (Benjamin, Golijanin et al. 1999; Folkman 2007). Consequently, the anti-tumor effect of angiogenic inhibitors was thought to differ from that of chemotherapy because it principally targets activated microvascular endothelial cells within the tumor bed rather than the tumor itself. However, there is currently wide debate over the exact mechanism of action of anti-angiogenic agents, and whether or not they are effective alternatives for the treatment of GBM.

1.3.1. Potential mechanisms of angiogenic inhibitor action

Many possible mechanisms have been proposed to describe the potential activity of angiogenic inhibitors in GBM. Firstly, because GBM tumor cells themselves have been found to express angiogenic receptors including VEGFR (Knizetova, Ehrmann et al. 2008), there remains a possibility that anti-angiogenic agents can have direct anti-tumor effects as well as effects on tumor vasculature. Additionally, anti-angiogenic treatment can potentially counteract the surge in VEGF secretion and/or accelerated tumor repopulation reported to occur following RT or chemotherapy in GBM (Hovinga, Stalpers et al. 2005; Shaked and Kerbel 2007). Furthermore, anti-angiogenic drugs have also been shown to preferentially target BTICs by exploiting their location in the brain. BTICs predominantly exist in a highly specialized perivascular niche, in which interactions with endothelial cells within the immediate microenvironment allow these cells to remain in a self-renewing and tumorigenic state (Calabrese, Poppleton et al. 2007). Therefore, anti-angiogenic therapy may suppress GBM tumorigenicity by directly targeting BTICs and by critically disturbing the perivascular niche, thereby further contributing to BTIC death (Bao, Wu et al. 2006; Gilbertson and Rich 2007).

However, the most commonly accepted theory of anti-angiogenic action is the process of vascular normalization. As described in section 1.1.3, the GBM tumor vascular network is highly disorganized and characterized by abnormal perfusion, resulting in inefficient chemotherapy delivery and diminished radiation sensitivity resulting from impaired oxygen delivery (Gerstner and Batchelor

2012). Treatment with angiogenic inhibitors can hypothetically prune immature and inefficient blood vessels by eliminating excess endothelial cells so that remaining vasculature is more "normal" and hence more conducive to conventional and new molecular-targeted chemotherapeutics (Jain 2001). In support of a normalization model, Abdollahi et al. showed that using a combination of a direct angiogenic inhibitor (endostatin) and a VEGFR-2 inhibitor in a GBM xenograft model resulted in reduced functional microvessel density, tumor circulation, and blood perfusion (Abdollahi, Lipson et al. 2003). Numerous *in vitro* and *in vivo* studies of GBM have also shown that concurrent anti-angiogenic therapy enhances RT and/or chemotherapy sensitivity as determined by reduced proliferative recovery, reduced clonogenic survival, increased apoptosis, and/or tumor growth inhibition (Bischof, Abdollahi et al. 2004; Damiano, Melisi et al. 2005; Quick and Gewirtz 2006; Combs, Schulz-Ertner et al. 2007). Notably, Winkler et al. (2004) found that treatment of orthotopic GBM xenografts with a VEGFR-2-specific monoclonal antibody creates a "normalization window" during which pericyte coverage of brain tumor vessels is increased and degradation of their abnormally thick basement membrane is induced. This window was also characterized by reduced tumor hypoxia and heightened radiosensitivity, thereby explicitly demonstrating that vascular normalization by anti-angiogenic agents can improve RT response in GBM (Winkler, Kozin et al. 2004). Clinical evidence supporting the vascular normalization hypothesis was also reported among recurrent GBM patients treated with the pan-VEGFR inhibitor AZD2171. Treatment induced rapid but transient

structural normalization of tumor vessels and was accompanied by a decrease in interstitial hypertension. As a result, patients had a reduced dependency on corticosteroid use (Batchelor, Sorensen et al. 2007). Similarly, Friedman et al. (2009) also reported that following treatment with angiogenic inhibitors, there was a trend for GBM patients taking corticosteroids to take a stable or decreasing dose overtime (Friedman, Prados et al. 2009), suggesting that anti-angiogenic treatment may also minimize the consequences of mass effect in GBM patients.

1.3.2. Notable angiogenic inhibitors for GBM

The most well developed anti-angiogenic agent to date is bevacizumab (Avastin). Bevacizumab is a humanized monoclonal immunoglobulin G_1 (IgG_1) antibody directed towards all active forms of VEGF. By binding VEGF, bevacizumab neutralizes its ability to bind to and activate VEGF receptors (Ferrara, Hillan et al. 2004; Shih and Lindley 2006). In 2004, bevacizumab, based on its efficacy in metastatic colorectal cancer, became the first angiogenic inhibitor to be approved by the Food and Drug Administration (FDA) (Hurwitz, Fehrenbacher et al. 2004). Since then bevacizumab has also been approved for use as a first line treatment for metastatic renal cell carcinoma and non-small-cell lung cancer, and as a second line treatment of metastatic colorectal cancer. (Heath and Bicknell 2009). In patients with recurrent GBM, when combined with irinotecan in phase II clinical trials, bevacizumab was shown to improve progression free survival (Vredenburgh, Desjardins et al. 2007; Friedman, Prados et al. 2009). Consequently, in May 2009 bevacizumab received accelerated FDA approval for

treatment of recurrent high-grade astrocytomas, making it the first drug to be approved for recurrent GBM in more than 10 years (Cohen, Shen et al. 2009), and only the third FDA approved chemotherapy for GBM in the past 37 years (Rose and Aghi 2010).

Despite the success of bevacizumab and other single-target angiogenic inhibitors, resistance invariably develops. These failures may result from the existence of multiple redundant or compensatory signaling pathways. Thus, simply targeting the VEGF pathway may not be sufficient for inhibiting angiogenesis in molecularly heterogeneous and aggressive tumors such as GBM. Indeed, combined treatment with PDGFR and VEGFR inhibitors was shown to enhance apoptosis, reduce cell proliferation and survival, increase tumor growth delay, and reduce endothelial cell migration and tube formation more effectively than either monotherapy in a preclinical study of GBM (Timke, Zieher et al. 2008). Therefore, the use of multi-targeted tyrosine kinase inhibitors (TKIs) in patients with GBM has gained attention in recent years. These small molecule inhibitors compete with adenosine-5'-triphosphate (ATP) for binding within the intracellular domain of various wild type and/or mutated RTKs (often including VEGFR), and therefore achieve a broader spectrum of activity than single-target agents. Because several RTKs implicated in growth and survival are often aberrantly expressed in endothelial cells of the tumor vasculature as well as the GBM tumor cells themselves, multi-targeted TKIs can target both tumor cells and surrounding supportive cells. The use of a single multi-targeted agent offers the additional benefit of reducing the number of drugs a patient is required to take,

which may consequently minimize drug-drug interactions and toxicity (Faivre, Demetri et al. 2007). The first two multi-targeted TKIs approved by the FDA for use as cancer therapies are sunitinib malate (Sutent, SU11248) and sorafenib tosylate (Nexavar).

Sunitinib is currently approved for treatment of renal cell carcinoma and gastrointestinal stromal tumors (Goodman, Rock et al. 2007). In addition to being a pan-VEGFR inhibitor (targeting VEGFR-1, -2, -3), sunitinib inhibits numerous other RTKs that are biologically relevant in GBM, such as PDGFR-α and –β, stem cell growth factor receptor (c-KIT) (Sakamoto 2004; Joensuu, Puputti et al. 2005; Anderson, McFarland et al. 2008), FMS-like tyrosine kinase 3 (FLT3) and colony stimulating factor 1 receptor (CSF1-R) (Potapova, Laird et al. 2006; Faivre, Demetri et al. 2007). Sunitinib has shown encouraging activity in a number of cancer models. In preclinical studies of GBM, sunitinib alone (de Bouard, Herlin et al. 2007) or in combination with RT (Schueneman, Himmelfarb et al. 2003) has shown potent anti-angiogenic and anti-invasive effects. In addition, in combination with TMZ, sunitinib was shown to enhance penetration of the alkylating agent into a GBM tumor xenograft (Zhou, Guo et al. 2008).

Sorafenib, an approved treatment for renal cell carcinoma and hepatocellular carcinoma, is also a promising therapeutic option for GBM. It was originally developed as an inhibitor of Raf, a key mediator of the Ras/Raf/MAPK signaling cascade commonly over activated in GBM, but was subsequently shown to inhibit multiple other kinases such as VEGFR-1 and -2, PDGFR (Wilhelm, Adnane et al. 2008), c-KIT, and FLT3 (Lierman, Lahortiga et al. 2007; Handolias,

Hamilton et al. 2010). Accordingly, sorafenib alone (Siegelin, Raskett et al. 2010; Yang, Brown et al. 2010) or in combination with pemetrexed (Bareford, Park et al. 2011) was shown to inhibit proliferation and induce autophagy in glioma cells *in vitro* and *in vivo*.

1.3.3. Resistance to anti-angiogenic therapy

Unfortunately, the benefits of such anti-angiogenic agents both in preclinical and clinical studies of GBM are at best transitory. Clinical trials in which progression free survival is the primary study endpoint tend to show promising results characterized by tumor stasis or shrinkage, but this brief period of clinical benefit is inevitably followed by tumor restoration and progression. Thus, in patients with GBM, treatment with bevacizumab, sunitinib, sorafenib, and other angiogenic inhibitors has failed to produce favorable enduring responses, with no significant extension of overall survival (Lai, Tran et al. 2011; Neyns, Sadones et al. 2011; Reardon, Vredenburgh et al. 2011a; Reardon, Vredenburgh et al. 2011b). Knowledge of the mechanistic basis underlying this resistance is important for future treatment using these drugs. Currently, the two proposed modes of resistance to angiogenic inhibitors (particularly those targeting VEGF and related pathways) are intrinsic and adaptive/evasive resistance (Figure 1.7).

A minority of patients treated with anti-angiogenic therapy fail to show any clinical benefit (Batchelor, Sorensen et al. 2007). Because of the absence of a period of tumor stasis, shrinkage, or retardation of growth rate, these tumors are

considered to possess intrinsic resistance to angiogenic inhibitors. One possible mechanism of intrinsic resistance may be due to a multidrug resistance phenotype characterized by increased extrusion of drugs through ATP-binding cassette membrane efflux pumps (Tews, Nissen et al. 2000).

Additionally, vascularization in GBM tumors that are refractory to anti-angiogenic therapy may not be dependent on angiogenesis, and therefore may not respond to VEGF inhibition. In support of this concept, mouse models of GBM showed evidence that highly infiltrative tumors can surround pre-existing vessels in the brain, thus enabling continued migration and growth (Sakariassen, Prestegarden et al. 2006). This vessel co-option is an efficient mechanism of resistance because these existing vessels do not exhibit angiogenesis and are therefore not targeted by angiogenic inhibitors (Verhoeff, van Tellingen et al. 2009). Human GBM tissues have also shown evidence of vasculogenic mimicry, in which non-endothelial cell-lined tubular channels formed by tumor cells are able to transport blood throughout the tumor (El Hallani, Boisselier et al. 2010; Liu, Zhang et al. 2011).

Furthermore, a population of BTICs may be responsible for continued development of tumor vasculature by angiogenesis or vasculogenesis regardless of VEGF pathway inhibition. Orthotopic injection of human BTICs in mice produced tumor xenografts containing vessels that were primarily composed of human endothelial cells (Ricci-Vitiani, Pallini et al. 2010), suggesting that tumor vasculature did not develop by classical angiogenesis (in which new vessels sprout from pre-existing vessels in the brain). Numerous studies have also shown

that subpopulations of BTICs have the ability to transdifferentiate into vascular smooth muscle-like cells (El Hallani, Boisselier et al. 2010) or endothelial progenitor cells (Wang, Chadalavada et al. 2010; Soda, Marumoto et al. 2011). Indeed, a variable number of endothelial cells in GBM carry the same genomic alterations as tumor cells, thereby indicating a neoplastic origin of tumor blood vessels. Importantly, though exposure to bevacizumab can inhibit the maturation of tumor endothelial progenitor cells into endothelium, it cannot inhibit the differentiation of BTICs into endothelial progenitors (Wang, Chadalavada et al. 2010). Therefore, the tumor can continually contribute to its own vasculature, and consequently its growth, despite inhibition from anti-angiogenic agents.

Because the majority of cases indicate initial transitory response to anti-angiogenic agents, tumor cells likely adapt to treatment by acquiring the means to functionally evade angiogenesis blockade. These evasive mechanisms reflect transcriptional changes that enable the tumor to grow while the specific therapeutic target of the anti-angiogenic agent remains inhibited (Bergers and Hanahan 2008). For instance, the activation of alternative pro-angiogenic signaling pathways to re-establish neovascularization may be one adaptive mechanism. Lucio-Eterovic et al. (2009) found that although *in vitro* bevacizumab treatment resulted in decreased expression of VEGF, GBM cells increased transcription and translation of several other pro-angiogenic factors (Lucio-Eterovic, Piao et al. 2009). Similarly, Batchelor et al. (2007) saw that in a clinical investigation of GBM patients treated with a pan-VEGFR inhibitor, the relapse and progression phase occurring after initial response was associated with higher

blood levels of FGF-2. This upregulation was accompanied by a re-initiation of angiogenesis and a loss of vascular normalization (Batchelor, Sorensen et al. 2007). Another distinct mechanism of evasive resistance exemplified in GBM is increased recruitment of vascular progenitor cells and bone marrow-derived cells (BMDCs) possessing the capacity to fuel tumors by producing new blood vessels through vasculogenesis (Du, Lu et al. 2008; Rahman, Smith et al. 2010).

However, it is becoming increasingly evident that the primary mode of evasion from angiogenic inhibition is an escalation of tumor invasiveness. This evasive mechanism was first described in an orthotopic GBM mouse model where tumor cells became more invasive and were seen to co-opt normal blood vessels following genetic deletion or pharmacological inhibition of VEGF. Invasive cells also appeared to migrate along normal vessels as multicellular layers rather than the normal mode of infiltration as single cells (Rubenstein, Kim et al. 2000). These preclinical observations were further validated when VEGF knockdown in GBM cells resulted in an upregulation of the invasive marker YKL-40 (Saidi, Javerzat et al. 2008), and when treatment with sunitinib was shown to increase *in vivo* GBM invasion (Ebos, Lee et al. 2009; Paez-Ribes, Allen et al. 2009). Keunen et al. (2011) further revealed that increased invasiveness seen in *in vivo* GBM models is a result of a hypoxic environment induced by anti-VEGF treatment, as indicated by the upregulation of HIF-1α and activation of the PI3K pathway leading to a metabolic shift in tumor cells (Keunen, Johansson et al. 2011). Importantly, numerous clinical studies have also reported that following treatment with angiogenic inhibitors, GBM patients can develop progressive disease

accompanied by a higher rate of diffuse, infiltrative lesions (Norden, Young et al. 2008; Iwamoto, Abrey et al. 2009; Narayana, Kelly et al. 2009; de Groot, Fuller et al. 2010; di Tomaso, Snuderl et al. 2011; Narayana, Gruber et al. 2012). These observations have dramatic clinical consequences, as GBM is already a highly invasive tumor that exhibits severe resistance to therapy. The possibility that anti-angiogenic treatment can exacerbate this phenotype necessitates a deeper evaluation of the optimal utilization of these agents in GBM. However, it is important to note that presentation of invasive relapse is not uniformly observed (Chamberlain 2011), suggesting that the heterogeneous nature of GBM might explain why certain individuals respond better than others.

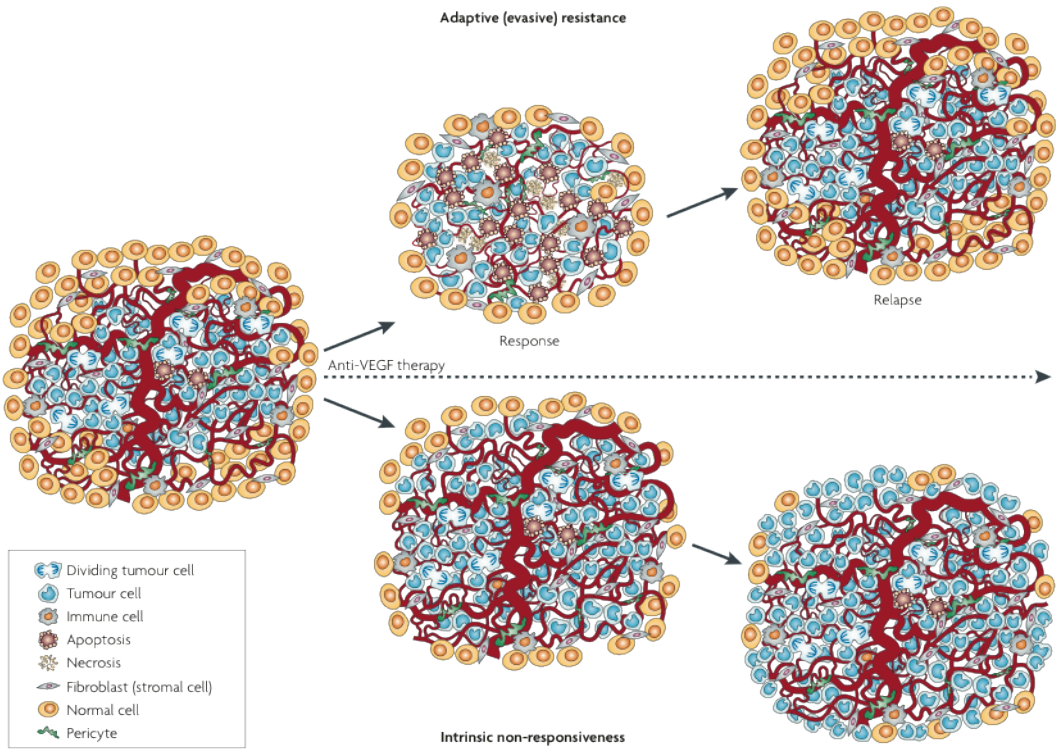

Figure 1.7. Two modes of resistance to anti-angiogenic therapy. Adaptive resistance refers to the ability of a tumor, after an initial response phase, to evade therapeutic blockade and renew tumor growth to progression. Conversely, intrinsic non-responsiveness is a pre-existing condition defined by the absence of any beneficial effect of anti-angiogenic therapy, resulting in unabated tumor growth. (Adapted from (Bergers and Hanahan 2008), *Nature Reviews. Cancer*)

1.4. RATIONALE AND HYPOTHESIS

The characterization of MGMT as an alkyltransferase has been extensively described in the context of response to alkylating agents, and it is thus far believed to be the exclusive function of MGMT. Accordingly, *MGMT* gene silencing by promoter methylation is correlated with a hypermutator phenotype. Thus, decreases in MGMT expression, and consequently activity, are associated with GBM tumor progression. However, normal brain tissue expresses low levels of MGMT while gliomas often display higher expression, which implies that increased MGMT expression may correlate with tumor progression. Therefore, the role of MGMT in GBM appears to be complex, and requires further investigation. Recent studies have found that MGMT expression is correlated with phenotypic alterations of several tumors, suggesting MGMT may play a more significant part in GBM beyond mediation of alkylating chemotherapy resistance.

Angiogenesis, invasion, proliferation, and MGMT status are key mediators of GBM resistance to therapy and recurrence. The purpose of this thesis is to establish a relationship between these key mediators, and to determine if MGMT can influence response to alternative therapy with angiogenic inhibitors. We hypothesize that MGMT influences the aggressive phenotype of GBM cells and mediates response to anti-angiogenic therapy. In our investigation we are primarily using U87MG and T98G GBM cell lines that are MGMT(-) and MGMT(+) respectively, their counterparts transfected with an MGMT-expressing construct and an MGMT shRNA construct (U87/MGMT and T98sh respectively), as well as panels of established and primary GBM cell lines.

1.6. REFERENCES

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Chapter 2: MGMT modulates glioblastoma angiogenesis and response to the tyrosine kinase inhibitor sunitinib

A version of this chapter has been published. **Chahal M**, Xu Y, Lesniak D, Graham K, Famulski K, Christensen JG, Aghi M, Jacques A, Murray D, Sabri S, Abdulkarim B. (2010). MGMT modulates glioblastoma angiogenesis and response to the tyrosine kinase inhibitor sunitinib. **Neuro-Oncology**, 12(8): 822-833.

2.1. SUMMARY

Angiogenesis inhibitors such as sunitinib represent a promising strategy to improve glioblastoma (GBM) tumor response. In this study, we used the O^6-methylguanine-DNA methyltransferase (MGMT)-negative GBM cell line U87MG stably transfected with *MGMT* (U87/MGMT) to assess whether MGMT expression affects the response to sunitinib. We showed that the addition of sunitinib to standard therapy [temozolomide (TMZ) + radiation therapy (RT)] significantly improved the response of MGMT(+), but not of MGMT(-), cells. Gene expression profiling revealed alterations in the angiogenic profile, as well as differential expression of several receptor tyrosine kinases targeted by sunitinib. MGMT(+) cells displayed higher levels of VEGFR-1 compared to U87/EV cells, whereas they displayed decreased levels of VEGFR-2. Depleting MGMT using O^6-benzylguanine suggested that the expression of these receptors was directly related to MGMT status. Also, we showed that MGMT expression was associated with a dramatic increase of soluble VEGFR-1/VEGFA ratio, thereby suggesting a decrease of bioactive VEGFA, which shifts the balance in favor of an anti-angiogenic profile. The reduced angiogenic potential of MGMT(+) cells is supported by: (i) the decreased ability of their secreted factors to induce endothelial tube formation *in vitro* and (ii) their low tumorigenicity *in vivo* compared to the MGMT(-) cells. Our study is the first to show a direct link between MGMT expression and decreased angiogenicity and tumorigenicity of GBM cells and suggests the combination of sunitinib and standard therapy as an alternative strategy for GBM patients with MGMT(+) tumors.

2.2. INTRODUCTION

Glioblastoma (GBM) is the most aggressive primary malignant brain tumor in adults. Recently, concomitant temozolomide (TMZ) and radiation therapy (RT) followed by adjuvant TMZ became the standard of care for GBM patients (Stupp, Mason et al. 2005). However, correlative studies showed that patients with tumors displaying O^6-methylguanine methyltransferase (*MGMT*) promoter methylation [i.e., MGMT(-)] were more likely to benefit from combined RT and TMZ, with a 2-year survival rate of 46% compared to 14% for patients with unmethylated *MGMT* tumors [i.e., MGMT(+)] (Hegi, Diserens et al. 2005). Indeed, the DNA repair protein MGMT is able to counteract the cytotoxic effects of TMZ by removing alkyl groups from the O^6-position of guanine (Denny, Wheelhouse et al. 1994; Kaina, Christmann et al. 2007; Stupp, Hegi et al. 2009). Thus, alternative strategies for patients with unmethylated *MGMT* promoters (unresponsive tumors) are required to improve their poor outcome.

Tumoral neovascularization is induced by tumor expression of proangiogenic growth factors (Jain, di Tomaso et al. 2007), and several growth factors and their cognate receptors are known to be overexpressed in GBM (Dunn, Heese et al. 2000). Most notably, vascular endothelial growth factor (VEGF) and its primary receptors VEGFR-1/Flt-1 and VEGFR-2/KDR/FLK-1 are increased in brain tumors, and are widely considered to be the principal mediators of glioma angiogenesis (Ferrara 2004; Byrne, Bouchier-Hayes et al. 2005; Fischer, Gagner et al. 2005). Sunitinib malate (Sutent, SU11248) is a multi-targeted receptor tyrosine kinase (RTK) inhibitor with anti-angiogenic activities. In addition to

inhibition of VEGFR-1/-2/-3, sunitinib inhibits several RTKs involved in GBM growth and neovascularization, including platelet derived growth factor receptors (PDGFR α and β), stem cell growth factor receptor (c-KIT) (Sakamoto 2004; Joensuu, Puputti et al. 2005; Anderson, McFarland et al. 2008), FMS-like tyrosine kinase 3 (FLT3) and colony stimulating factor 1 receptor (CSF1-R) (Faivre, Demetri et al. 2007). In preclinical studies, sunitinib alone (de Bouard, Herlin et al. 2007) or in combination with RT (Schueneman, Himmelfarb et al. 2003) has shown potent anti-angiogenic and anti-invasive effects in GBM cell lines. Sunitinib is currently being tested in a phase II study of recurrent GBM. However, the effect of sunitinib in combination with TMZ+RT has not yet been investigated.

Genomic characterization of GBM tumors highlighted the association between *MGMT* promoter methylation and a hypermutator phenotype that encompasses global changes in DNA methylation and mutations in several genes (2008). These alterations would affect functional pathways dictating both tumor behavior and clinical response to TMZ or other drugs. We hypothesized that a combination of anti-angiogenic drugs with TMZ+RT must be evaluated in the context of MGMT status, which is so far the only available predictive biomarker of response to TMZ+RT. Thus, we first aimed to investigate cellular effects of sunitinib-based therapy in 3 GBM cell lines with differing MGMT status, including the highly tumorigenic and angiogenic MGMT(-) GBM cell line U87MG and its counterpart stably transfected with *MGMT* (U87/MGMT). The addition of sunitinib to TMZ+RT significantly improved the anti-proliferative

effects in these MGMT(+) compared to MGMT(-) cells. Additionally, gene expression profiling revealed for the first time that MGMT expression induced gene alterations involved in several functional pathways. Importantly, MGMT expression elicited a switch of the angiogenic balance toward an anti-angiogenic profile in a GBM background. We specifically show an association between high MGMT expression and decreased expression of VEGFR-2 and secretion of VEGFA, as opposed to increased levels of VEGFR-1 and its soluble form (sVEGFR-1).

These findings highlight a novel role of MGMT as a critical upstream regulator of genes involved in angiogenesis of GBM tumor cells. Accordingly, we believe that MGMT status should be assessed in future clinical trials testing anti-angiogenic therapy with TMZ+RT, which represents a promising strategy for patients with MGMT(+) tumors that are resistant to TMZ.

2.3. MATERIALS AND METHODS

2.3.1. Cell culture

The T98G GBM cell line was obtained from American Type Culture Collection. U87MG empty vector (U87/EV) and its MGMT-transfected derivative U87/MGMT (Aghi, Rabkin et al. 2006) cells were grown at 37ºC/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S) (Invitrogen) (standard media). The HMEC-1 endothelial cell line was obtained from Dr. Edmund Ades (Ades, Candal et al. 1992) (Center for Disease Control, GA) and was cultured in MCDB medium supplemented with 10% FCS and 1% P/S.

2.3.2. In vitro drug exposures

Sunitinib malate (Pfizer) and temozolomide (TMZ) (Schering-Plough) were dissolved in dimethylsulfoxide (DMSO). Cells were serum-starved in DMEM containing 0.5% FCS overnight, then exposed to sunitinib (1 μM) for 2 h, TMZ (100 μM) for 3 h in serum-starved media, and/or exposed to 4 Gy of ^{60}Co gamma radiation at a dose rate of 0.3 krad/min. MGMT was depleted with 20 μM O^6-Benzylguanine (O6BG, Sigma) dissolved in DMSO as previously described (Bobola, Silber et al. 2005). Cells were maintained in media containing O6BG during, and for 24 h after sunitinib treatment to ensure continued suppression of MGMT.

2.3.3. Proliferation and clonogenic survival assays

Cells growing at 70% confluency were treated as described above, harvested, and seeded in triplicate in a 96-well plate at a density of 5 x 10^2 cells/well (U87/MGMT, T98G) for 48 h, or 1 x 10^3 cells/well (U87/EV) for 72 h. Cellular proliferation was assessed using the XTT Cell Proliferation Kit (Roche Pharmaceuticals).

Clonogenic survival analysis was performed as described previously (Abdulkarim, Sabri et al. 2003). Briefly, cells plated at various densities (2 x 10^2 to 4.5 x 10^3) were allowed to adhere overnight and then treated as described above. After 10-14 days, cells were stained with 1% crystal violet and colonies with >50 cells were counted manually. Surviving fraction was calculated as follows: (colonies formed/total cells plated)/plating efficiency (as determined by DMSO control plates).

2.3.4. Enzyme-linked immunosorbent assay (ELISA)

Cells growing at 70% confluency were treated as described above. After treatment, the media was replaced with standard media for an additional 24 h. The resultant conditioned medium was collected and passed through a 0.22 μm filter to remove cell debris. VEGF and sVEGFR-1 ELISA analyses (R&D Systems) were performed according to the manufacturer's instruction. The VEGFA and sVEGFR-1 concentrations were calculated from standard curves generated using recombinant human VEGFA and recombinant human VEGFR-1, respectively.

2.3.5. Western blot analysis

Following treatment, cells were washed twice with phosphate-buffered saline (PBS) and lysed with RIPA buffer (20 nM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM NaPP, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin) (Schueneman, Himmelfarb et al. 2003). 30 μg of protein (BCA protein assay kit, Pierce) was separated by 12% SDS-PAGE under reducing conditions and transferred onto polyvinylidine difluoride membranes. Membranes were probed for phospho-Akt (Cell Signaling), Akt1/2/3 (Santa Cruz), phospho-ERK1/2 (Cell Signaling), ERK1/2 (Cell Signaling), β-actin (Sigma-Aldrich), and human MGMT (BD Biosciences) as previously described (Lokker, Sullivan et al. 2002). Subsequently, membranes were washed, and incubated with horseradish-peroxidase conjugated secondary antibody for 1 h. The protein expression was visualized using an enhanced chemiluminescent detection system (Amersham Biosciences AB).

2.3.6. Tumor growth in mice

A total of 5 x 10^6 cells (150 μL) were injected subcutaneously into the right flank of Balb/c, NIH III, or CDI/nu nude mice. Tumor growth was monitored twice a week using a digital caliper. Volume of tumors measuring >3 mm in diameter was calculated by the formula: Tumor volume = width^2 (short axis) × length (long axis) x 0.5, as described (Huang, Allam et al. 1995).

2.3.7. Gene expression microarray studies

Total RNA was isolated from independent cell samples in triplicate using Trizol (Sigma-Aldrich) and purified using Qiagen RNeasy columns (Qiagen) according to the manufacturer's instructions. The RNA was quantified using a NanoDrop 1000 Spectrophotometer and its integrity evaluated using a Bioanalyzer 2100 (Agilent) according to the manufacturer's protocols. The RNA was subjected to linear amplification and Cy3 labeling followed by hybridization to Agilent Whole Genome Arrays. Agilent kits were used according to the manufacturer's recommended protocols. Arrays were scanned using an Agilent Scanner, the data were extracted and the quality evaluated using Feature Extraction Software 9.5 (Agilent). The data were normalized and only the entities flagged as being present or marginal in at least 3 samples were included in the analysis (GeneSpring GX 10, Agilent). Genes that were more than two-fold up- or down-regulated (P values <0.05, unpaired Student's t-Test with Bonferroni multiple testing correction) were identified. Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da, Sherman et al. 2009) was used to identify enriched Gene Ontology (GO) biological themes (Ashburner, Ball et al. 2000). The GO data mining was conducted at a term specificity level 3 (Dennis, Sherman et al. 2003). The EASE score was set at 0.05 and the minimum number of genes in a category was five.

2.3.8. Quantitative real-time PCR (QRT-PCR)

RNA was extracted from 1 x 10^6 cells with the RNeasy mini-kit (Qiagen). Briefly, 1 μg of total RNA was reverse transcribed into cDNA using the superscript reverse transcription kit (Invitrogen), and cDNA was quantified by QRT-PCR on an ABI 9700HT system (Applied Biosystems). RT-PCR reactions were done using SYBR green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Analysis was performed using the comparative C_T method (Schmittgen and Livak 2008).

Primer sequences were as follows: VEGFR-1 sense, 5′-CTCTACTCCTGAAATCTATCAGA-3′; antisense, 5′-TACCATCCTGTTGTACATTTGCT-3′; VEGFR-2 sense, 5′-ACACCAGAAATGTACCAGACCAT-3′; antisense, 5′-TGCCATCCTGCTGAGCATTAG-3′; GAPDH sense, 5′-TCGCCAGCCGAGCCACAT-3′; antisense, 5′-CAATACGACCAAATCCGTTGACT -3′.

2.3.9. Flow cytometry analysis

Cells (1 x 10^6) were harvested, washed twice with PBS and fixed with 2% paraformaldehyde for 30 min. Cells were washed three times and Phycoerythrin (PE)-conjugated anti-VEGFR-1 or anti-VEGFR-2 (1:100) was added for 30 min. PE-conjugated IgG_1 was used as a negative control. Cells were washed three times and suspended in 0.5 mL of PBS and 10,000 events were acquired on a BD

FACScalibur flow cytometer. Results were analyzed using Cell Quest software (BD Biosciences).

2.3.10. Endothelial tube cell formation assay

24-well plates were coated with 300 μL of matrigel (BD Biosciences) for 30 min at 37ºC. 2 x 10^5 HMEC-1 cells in 1 mL of U87/EV or U87/MGMT conditioned media (1:2 dilution with serum-starved DMEM) were added to each well. Conditioned medium was collected following 48 h of culture in standard medium. After 12 h, cells were stained with 8 μg/mL calcein AM (Invitrogen) for 30 min at 37ºC (Di Simone, De Santis et al. 2007). Endothelial tube structures were examined using a Zeiss LSM 510 Axiovert 100M microscope with a Fluar Zeiss 5x 0.25 NA lens. MetaMorph 7.6 software was used to assess mean tube length, mean tube area, and number of nodes.

2.4. RESULTS

2.4.1. Sunitinib-based treatment preferentially inhibits the proliferation and survival of MGMT(+) cells

To investigate whether sunitinib in combination with the standard therapy would modulate the cellular response of MGMT(-) and MGMT(+) GBM cell lines, we used the MGMT(-) cell line U87MG (U87/empty vector, U87/EV) and its derived clone stably transfected with *MGMT* (U87/MGMT) (Aghi, Rabkin et al. 2006). As shown by immunoblotting, U87/MGMT and T98G cells, which exhibit constitutive expression of MGMT, had increased levels of MGMT protein compared to U87/EV cells (Figure 2.1A).

Based on our data and previous reports (Schueneman, Himmelfarb et al. 2003; de Bouard, Herlin et al. 2007), sunitinib at 1 μM was associated with significant inhibition of cell proliferation and was ultimately selected for subsequent studies. First, we used the XTT assay to test the efficacy of combining sunitinib (1 μM) with TMZ (100 μM) and/or RT (4 Gy) on cellular proliferation (Figure 2.1B). As expected, compared to RT alone, TMZ alone and the combination of TMZ+RT significantly decreased the proliferation of U87/EV cells ($P = 0.003$; $P < 0.001$, respectively), but not of U87/MGMT cells ($P = 0.7$; $P = 0.7$, respectively). Sunitinib alone or in combination with RT, TMZ, or TMZ+RT did not significantly decrease the proliferation of U87/EV cells compared to the same treatments without sunitinib. In contrast, the addition of sunitinib to each treatment decreased the proliferation of U87/MGMT cells to a

greater extent than RT alone ($P = 0.02$), TMZ alone ($P = 0.03$), or TMZ+RT ($P = 0.007$).

Next, we assessed the ability of sunitinib-based therapy to inhibit clonogenic survival of MGMT(-) and MGMT(+) cells (Figure 2.1C). A drastic loss of colony forming ability occurred when U87/EV cells were treated with the combination of TMZ+RT when compared to RT alone ($P = 0.004$), but this effect was not seen with U87/MGMT cells ($P = 0.3$). As shown in the proliferation assay, the effect of sunitinib alone was more pronounced on U87/MGMT compared to U87/EV cells ($P = 0.01$). Interestingly, the combination of sunitinib with RT, TMZ, or TMZ+RT significantly decreased the surviving fraction of U87/MGMT cells when compared to RT alone ($P = 0.04$), TMZ alone ($P < 0.001$) or TMZ+RT ($P = 0.02$). In contrast, the addition of sunitinib to RT and/or TMZ in MGMT(-) U87/EV cells did not further inhibit cell survival compared to RT and/or TMZ alone. Furthermore, proliferation and clonogenic survival of the MGMT(+) cell line T98G were also significantly inhibited by sunitinib in combination with TMZ+RT (Figure 2.2). These data suggest that the addition of sunitinib preferentially improved the response of MGMT(+) cells to standard therapy.

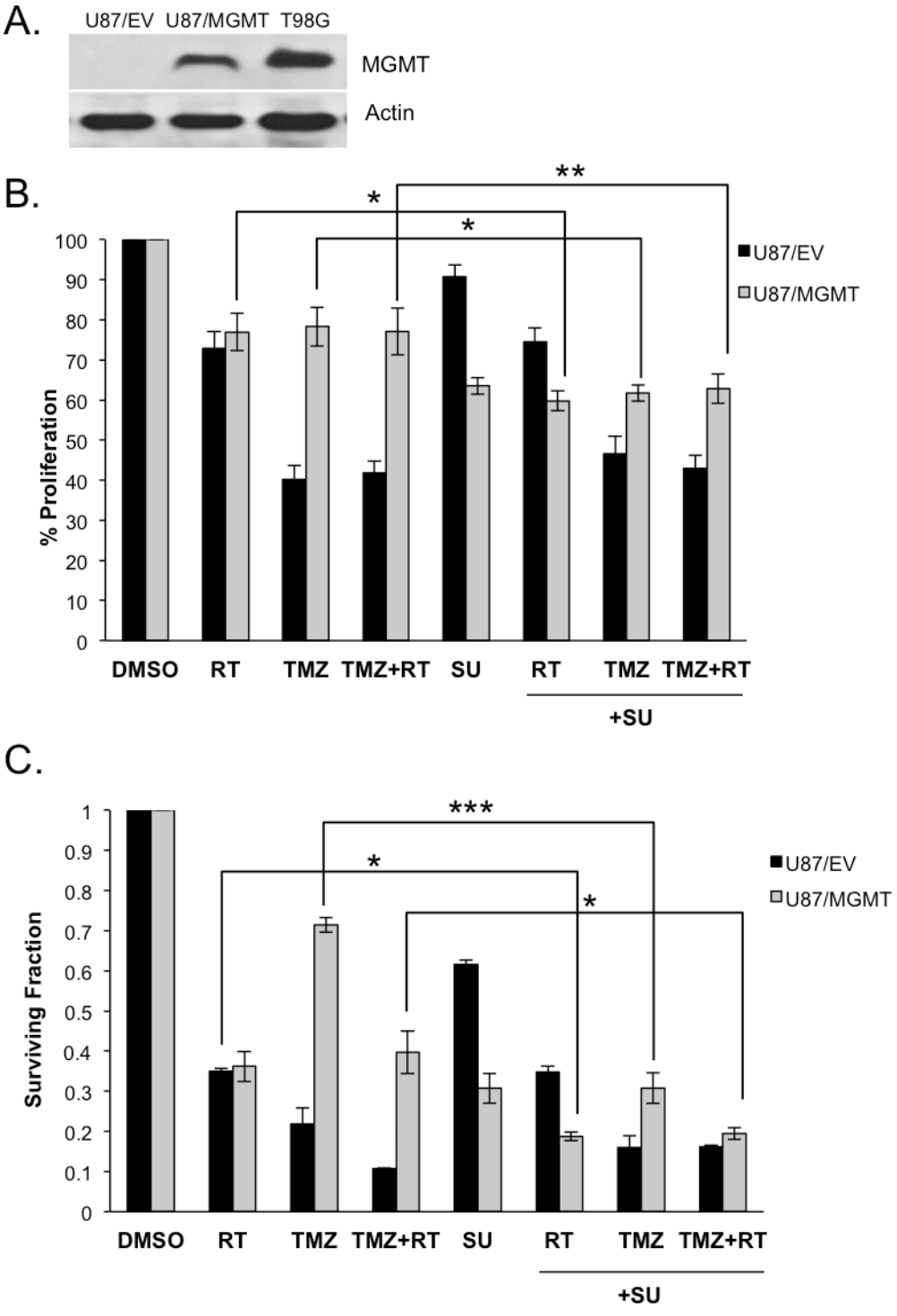

Figure 2.1. Sunitinib-based treatment decreases proliferation and survival of MGMT(+) cells. (A) MGMT expression determined by immunoblotting, **(B)** Effect of sunitinib (SU, 1 μM), RT (4 Gy) and/or TMZ (100 μM) on proliferation and **(C)** clonogenic survival of U87/EV and U87/MGMT cells. Mean values are normalized to a DMSO control. Error bars represent the SEM of at least 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

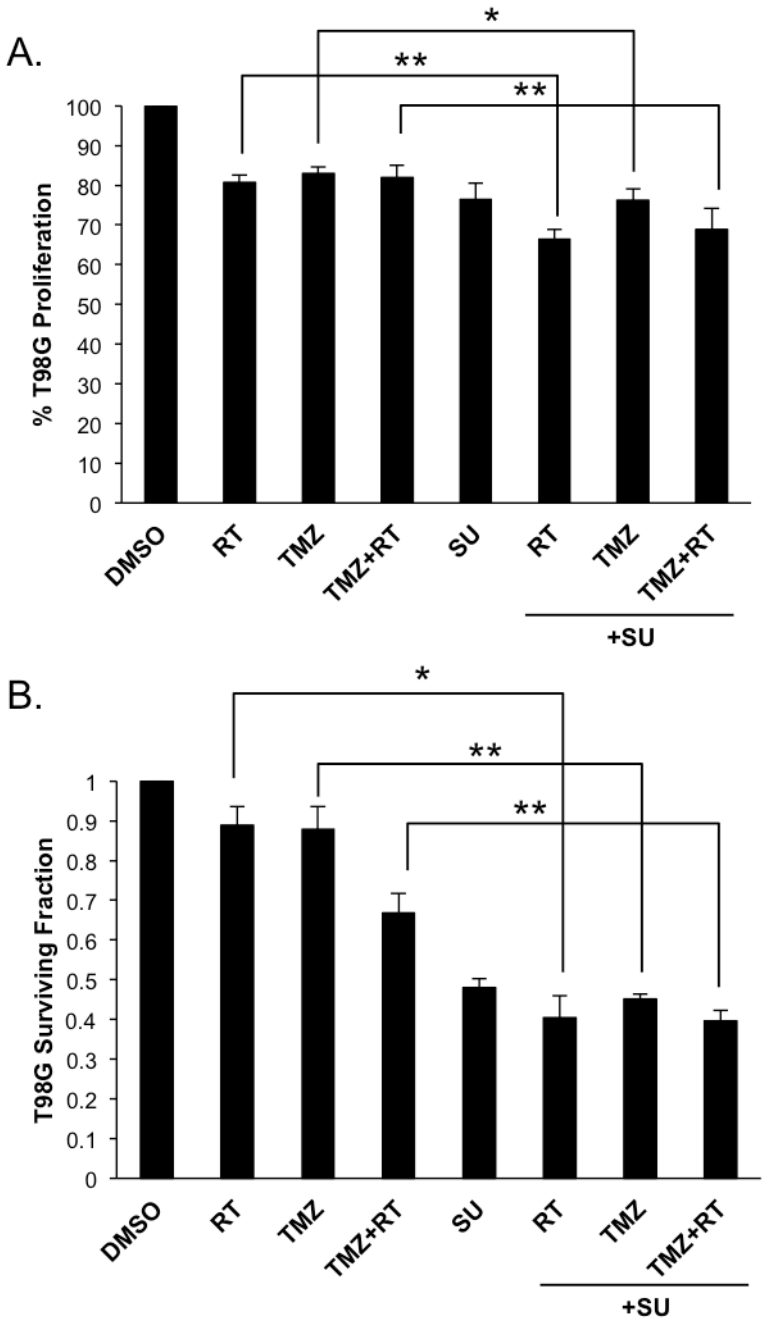

Figure 2.2. Sunitinib-based treatment decreases the proliferation and survival of MGMT(+) T98G cells. Effect of sunitinib (SU, 1 μM), radiation (RT, 4 Gy) and/or temozolomide (TMZ, 100 μM) on **(A)** proliferation and **(B)** clonogenic survival of T98G cells. Mean values are normalized to a DMSO control. Error bars represent the SEM of at least 3 independent experiments. *$P < 0.05$, **$P < 0.01$.

2.4.2. Sunitinib inhibits ERK1/2 and Akt phosphorylation in MGMT(+) cells

ERK1/2 and Akt, signaling molecules primarily involved in cell proliferation and survival, are downstream of several angiogenic growth factor receptors including those targeted by sunitinib (such as VEGFRs) (Byrne, Bouchier-Hayes et al. 2005). To assess the effect of sunitinib alone and in combination with standard therapy on these downstream kinases, we analyzed the phosphorylation of ERK1/2 and Akt by immunoblotting 24 h after drug exposure. Compared to DMSO control, sunitinib alone and sunitinib-based treatment (with RT, TMZ, or TMZ+RT) induced a marked decrease of Akt-Ser473 phosphorylation in U87/MGMT cells, but not in U87/EV cells. Similarly, sunitinib-based treatment (RT and/or TMZ) also decreased ERK1/2 phosphorylation in U87/MGMT cells but not in U87/EV cells (Figure 2.3A and B).

To investigate whether the inhibitory effect of sunitinib on ERK1/2 and Akt phosphorylation in MGMT(+) cells was related to MGMT status, we depleted MGMT protein levels using O6BG, a substrate analogue of MGMT which induces MGMT degradation (Dolan, Moschel et al. 1990). Compared to DMSO control, O6BG (20 μM) depleted MGMT expression in U87/MGMT cells by 90%, i.e., to a level comparable to U87/EV cells. Sunitinib treatment did not affect MGMT levels, but it decreased the phosphorylation of both Akt and ERK1/2 by 50%. In contrast, depletion of MGMT using O6BG in cells treated with sunitinib completely abrogated the inhibitory effect of sunitinib on the phosphorylation of ERK1/2 and partially blocked the dephosphorylation of Akt

(Figure 2.3C). Treatment with O6BG did not significantly affect the proliferation of both MGMT(+) cell lines (U87/MGMT and T98G). As expected, compared to either DMSO or O6BG treatment, sunitinib treatment significantly decreased the proliferation of U87/MGMT ($P < 0.001$ and $P < 0.001$, respectively) and T98G cells ($P < 0.001$ and $P = 0.006$, respectively). Interestingly, the addition of O6BG to sunitinib decreased the anti-proliferative effects of sunitinib to similar levels as for O6BG treatment alone in both U87/MGMT and T98G cells ($P = 0.16$ and $P = 0.15$, respectively) (Figure 2.3D). Our data suggest that the anti-proliferative effect of sunitinib on U87/MGMT and T98G cells is mediated through decreased phosphorylation of ERK1/2 and Akt, and that this inhibitory effect is related to MGMT expression in these cells.

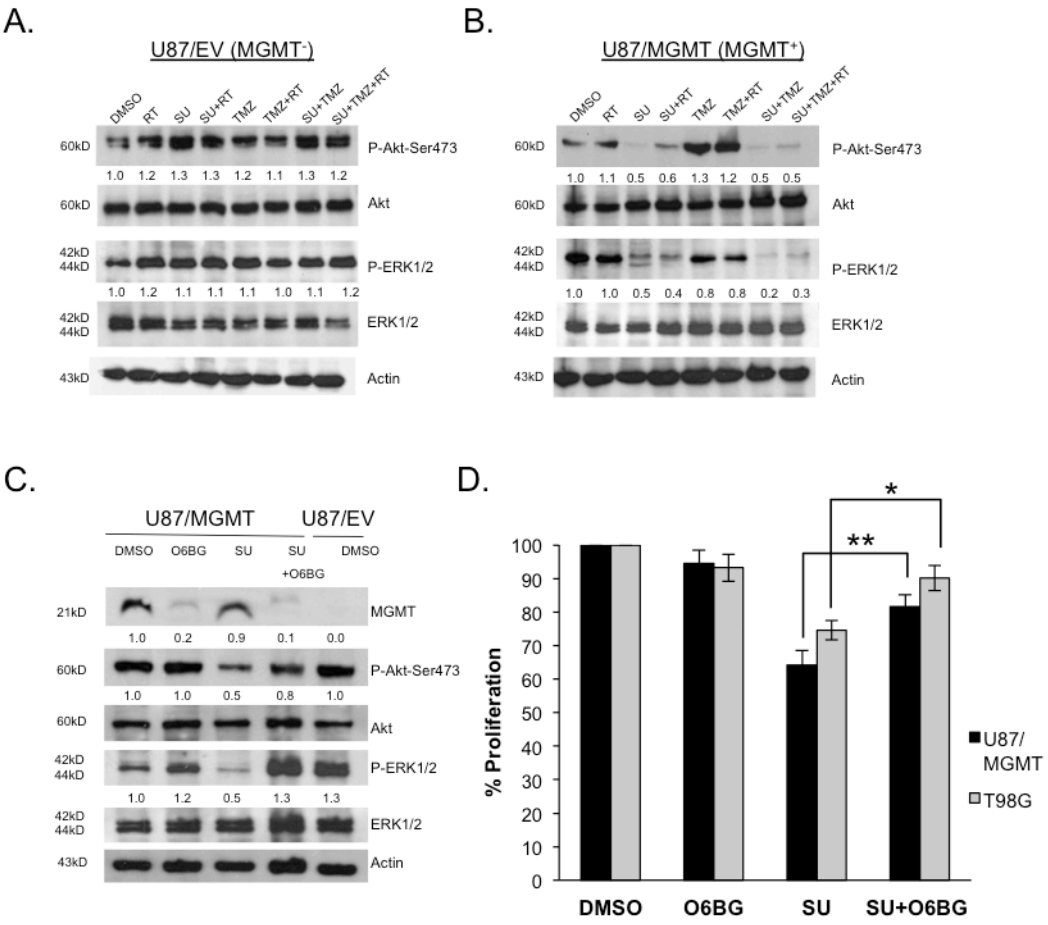

Figure 2.3. Sunitinib inhibition of ERK1/2 and Akt phosphorylation is dependent on MGMT status. (A) Phosphorylation of ERK1/2 and Akt-Ser473 in response to sunitinib (SU, 1 μM), RT (4 Gy) and/or TMZ (100 μM) in U87/EV cells and **(B)** in U87/MGMT cells. **(C)** Phosphorylation of ERK1/2 and Akt-Ser473 in U87/MGMT in response to sunitinib following O6BG (20 μM). DMSO-treated U87/EV cells were used as a negative control. Values below bands represent relative intensities (histogram analysis using Adobe Photoshop) normalized to their respective total forms and the DMSO control conditions. Experiments were repeated twice with similar results. **(D)** Proliferation of U87/MGMT and T98G cells treated with O6BG and/or sunitinib. Mean values are normalized to the DMSO control. Error bars represent the SEM of 3 independent experiments. $*P < 0.05$, $**P < 0.01$.

2.4.3. Reduced tumorigenic potential of MGMT(+) U87/MGMT cells

To compare the tumorigenicity of U87/EV and U87/MGMT cells, Balb/c *nu/nu* mice were subcutaneously injected with both cell lines, one in each flank. Whereas U87/EV cells rapidly generated tumors in this mouse xenograft model, the growth and sustainability of U87/MGMT tumors was drastically suppressed up to 9 weeks post-injection (Figure 2.4). Because growth factor-reduced matrigel has been shown to enhance the tumorigenicity of GBM cell lines *in vivo* (Mullen 2004), subcutaneous injection of U87/MGMT cells in a matrigel vehicle was attempted. Although palpable tumors did form initially, they spontaneously regressed within two weeks even when other mouse genetic backgrounds (CDI/nu or NIH III) were used as recipients. Orthotopic injection of U87/MGMT cells also did not show evidence of tumor growth in necropsy studies (P. Forsyth unpublished data). Additionally, among several MGMT-transfected U87 clones, only those expressing lower levels of MGMT were able to form xenografts (M. Aghi, unpublished data). Another MGMT(+) cell line, T98G, previously reported to be poorly tumorigenic (Rubenstein, Shaw et al. 1999), also showed decreased tumorigenicity in our model (Table 2.1). These results reveal for the first time a link between MGMT expression and reduced tumorigenicity of GBM xenografts.

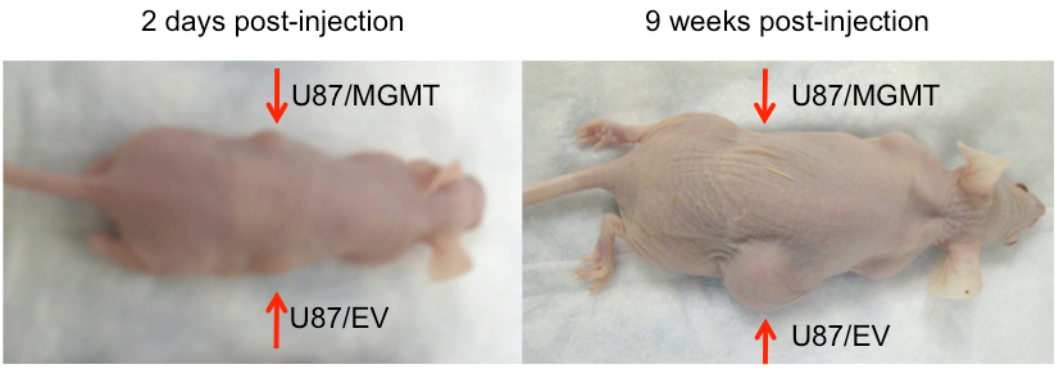

Figure 2.4. Tumor growth analysis of U87/EV and U87/MGMT xenografts. 3 Balb/c nu/nu mice were injected subcutaneously with 5 x 10^6 U87/EV cells (right flank) or U87/MGMT cells (left flank) with Matrigel. *Left*, Masses were apparent at the injection sites 2 days following injection. *Right,* 9 weeks following injection there was no evidence of a tumor at the U87/MGMT injection site, but U87/EV cells formed a large tumor.

Table 2.1. Tumorigenicity of MGMT(-) and MGMT(+) cell lines.

Tumor cell line	Injection vehicle	Mouse strain	# Mice injected	Greatest average tumor volume reached		
				# Mice with tumors	Mean ± SEM (mm^3)	Days post implantation
U87/EV	serum free media	Balb/c	55	42	2780.75 ± 39.57	45
U87/MGMT	serum free media	Balb/c	53	1	47.5	6
U87/MGMT	full serum media	Balb/c	6	3	< 5 ± 1	5
U87/MGMT	50% matrigel/ starved media	Balb/c	5	2	54.36 ± 22.95	8
U87/MGMT	50% matrigel/ starved media	NIH III	3	2	12.65 ± 10.51	8
U87/MGMT	50% matrigel/ starved media	CDI/nu	12	2	55.85 ± 19.61	8
T98G	serum free media	Balb/c	6	0	0	> 90
T98G	50% matrigel/ starved media	CDI/nu	3	0	0	> 90

2.4.4. Genes involved in angiogenesis are differentially regulated in MGMT(+) cells

To further investigate the differential response of U87/EV and U87/MGMT cells to sunitinib and the decreased *in vivo* tumorigenicity of U87/MGMT cells, we compared the expression of genes in U87/MGMT versus U87/EV cells by cDNA microarray. We identified 3,242 genes that were significantly differentially expressed by a minimum fold change of 2 (>99% CI, *P*-value fixed at <0.005, Student's *t* test). We used the Gene Ontology (GO) Consortium to classify genes into functional groups on the basis of biological process categories (Ashburner, Ball et al. 2000). GO analysis revealed that several functional pathways not previously related to the known functions of the MGMT protein were affected (Figure 2.5). These observations will likely set the stage for further investigations to validate the expression of some of these genes and unravel how MGMT affects their expression.

We also investigated genes that could potentially affect GBM angiogenesis and/or tumorigenicity and the response to sunitinib therapy. GO analysis revealed that genes involved in vasculature development and RTK signaling pathways (Table 2.2) were differentially regulated in U87/MGMT cells compared to U87/EV cells (P = 0.0001 and 0.0004, respectively). Interestingly, the expression of known sunitinib targets such as colony stimulating factor 1 receptor (*CSF1R*) and *VEGFR-2* were decreased in U87/MGMT cells. Additionally, expression of *VEGFA*, the most potent stimulator of angiogenic signaling (Park, Keller et al. 1993) and a key determinant of angiogenicity and

tumorigenicity of U87MG cells (Cheng, Huang et al. 1996), was also decreased in U87/MGMT cells. In contrast, expression of *VEGFR-1*, whose encoded protein exhibits ten-fold higher affinity for VEGFA than VEGFR-2 but has weaker tyrosine kinase activity (Park, Chen et al. 1994), was increased in U87/MGMT cells. Platelet-derived growth factor receptors (PDGFRs), well known targets of sunitinib (Sakamoto 2004), were not differentially regulated in U87/MGMT cells. Thus, the gene expression analysis revealed a dramatic switch in the angiogenic profile of U87/MGMT compared to parental cell line.

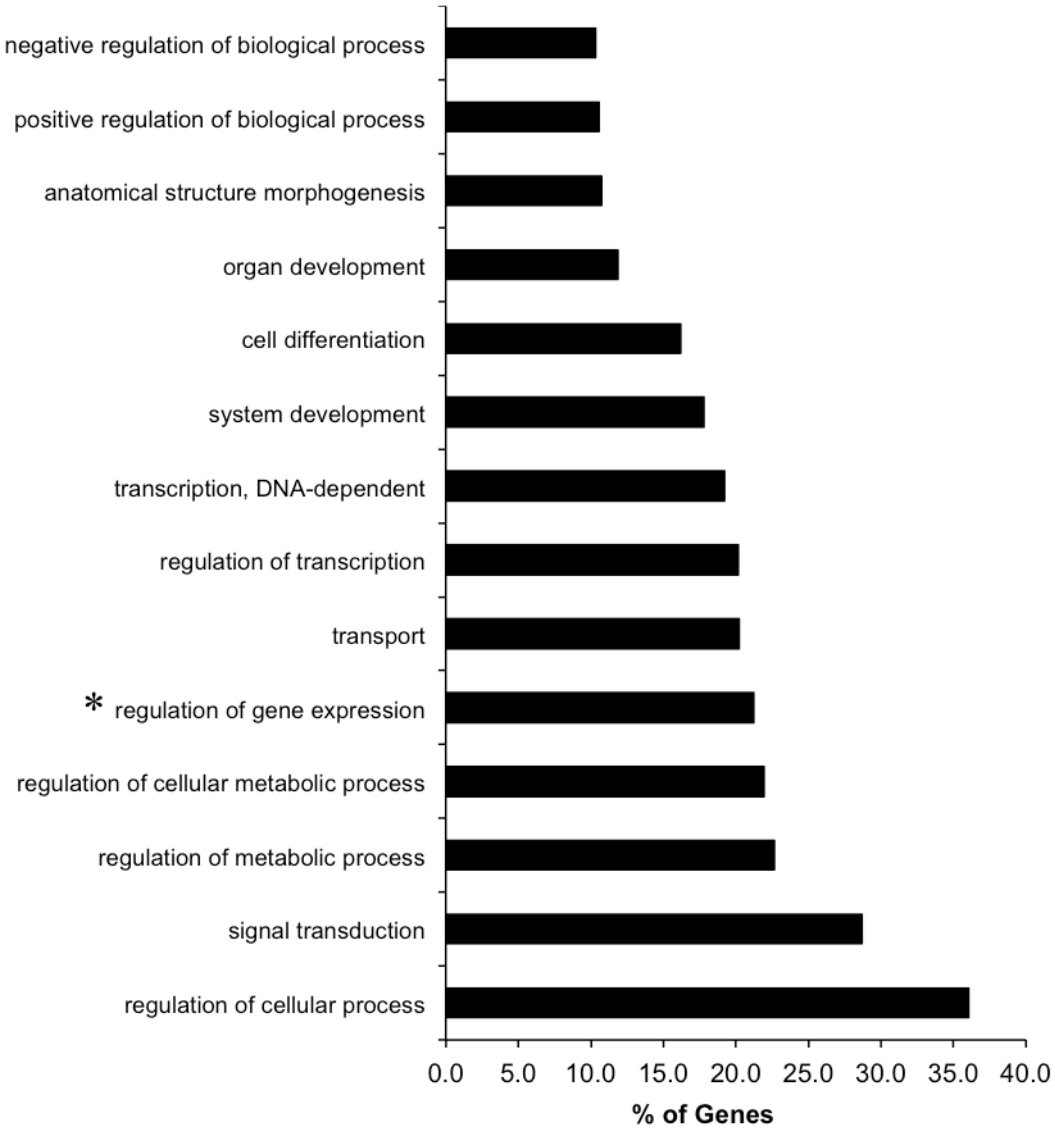

Figure 2.5. Gene Ontology data mining of U87/EV versus U87/MGMT cells. The 3,242 differentially expressed genes were characterized according to their biological process classification (at level 3) in the GO database. Genes were categorized into 63 GO classifications, and the graph depicts the classifications in which more than 10% of the genes are categorized. [*] This class contains several genes encoding zinc finger proteins.

Table 2.2. Differential expression of genes involved in angiogenesis and sunitinib response in U87/EV and U87/MGMT.

Gene	Symbol	U87/MGMT vs U87/EV Fold Change	GenBank
Biological Processes			
Vasculature development			
forkhead box F2; synonyms	FOXF2	**296.4**	NM_001452
angiotensinogen	AGT	**134.5**	NM_000029
endothelin 1	EDN1	**65.38**	NM_001955
EGF-like-domain, multiple 7	EGFL7	**55.31**	NM_201446
forkhead box F1	FOXF1	**24.54**	NM_001451
angiomotin	AMOT	**17.76**	NM_133265
laminin, alpha 4	LAMA4	**16.68**	NM_002290
vascular endothelial growth factor receptor 1	FLT1	**12.44**	NM_002019
carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1	**11.9**	NM_001712
7-dehydrocholesterol reductase	DHCR7	**8**	NM_001360
Homo sapiens tumor necrosis factor superfamily, member 12	TNFSF12	**7.738**	NM_153012
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	**6.798**	NM_001005862
fibroblast growth factor 18	FGF18	**5.937**	NM_033649
fibroblast growth factor 2	FGF2	**3.708**	NM_002006
c-fos induced growth factor (vascular endothelial growth factor D)	FIGF	**3.645**	NM_004469
B-cell translocation gene 1, anti-proliferative	BTG1	**3.54**	NM_001731
nuclear receptor subfamily 2, group F, member 2	NR2F2	**-2.35**	NM_021005
RAS p21 protein activator (GTPase activating protein) 1	RASA1	**-2.63**	NM_002890
fibroblast growth factor receptor 1	FGFR1	**-3.062**	NM_023111

Gene	Symbol	U87/MGMT vs U87/EV Fold Change	GenBank
Biological Processes			
Vasculature development (cont.)			
lysyl oxidase	LOX	**-3.66**	NM_002317
vascular endothelial growth factor A	VEGFA	**-5.33**	NM_001025366
alternatively spliced product of the AML1 gene	AML1	**-5.917**	D43967
Homo sapiens acid fibroblast growth factor-like protein	GLIO703	**-6.849**	AF211169
phosphatidic acid phosphatase type 2B	PPAP2B	**-9.615**	NM_003713
neuroplanin 2	NRP2	**-12.66**	NM_201266
Rho GTPase activating protein 22	ARHGAP22	**-13.53**	NM_021226
vascular endothelial growth factor receptor 2	KDR	**-16.73**	NM_002253
SRY (sex determining region Y)-box 17	SOX17	**-23.641**	NM_022454
plexin domain containing 1; tumor endothelial marker 7	PLXDC1	**-32.89**	NM_020405
serpin peptidase inhibitor, clade E, member 1	SERPINE1	**-111.48**	NM_000602
epiregulin	EREG	**-173.21**	NM_001432
integrin, alpha 7	ITGA7	**-181.49**	NM_002206
chondroitin sulfate proteoglycan 4	CSPG4	**-207.04**	NM_001897
podoplanin	PDPN	**-228.83**	NM_198389
Transmemebrane receptor protein tyrosine kinase signaling pathway			
adrenergic, beta-2-, receptor, surface	ADRB2	**138.7**	NM_000024
leukocyte tyrosine kinase	LTK	**90.08**	NM_002344

Gene	Symbol	U87/MGMT vs U87/EV Fold Change	GenBank
Biological Processes			
Transmemebrane receptor protein tyrosine kinase signaling pathway (cont.)			
fibroblast growth factor receptor 3	FGFR3	**65.97**	NM_000142
ephrin-A1	EFNA1	**39.62**	NM_004428
neurturin	NRTN	**36.63**	NM_004558
actin filament associated protein 1-like 2	AFAP1L2	**25.48**	NM_032550
vascular endothelial growth factor receptor 1	FLT1	**12.44**	NM_002019
ephrin-A4	EPHA4	**7.623**	NM_004438
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	**6.798**	NM_001005862
fibroblast growth factor 18	FGF18	**5.937**	NM_033649
c-fos induced growth factor (vascular endothelial growth factor D)	FIGF	**3.645**	NM_004469
Cas-Br-M (murine) ecotropic retroviral transforming sequence	CBL	**3.572**	NM_005188
SNF1-like kinase 2	SNF1LK2	**2.68**	NM_015191
eukaryotic translation initiation factor 2-alpha kinase 3	EIF2AK3	**2.197**	NM_004836
PTK2 protein tyrosine kinase 2; focal adhesion kinase 1	PTK2	**-2.246**	NM_153831
met proto-oncogene (hepatocyte growth factor receptor)	MET	**-2.397**	NM_000245
fibroblast growth factor receptor 1	FGFR1	**-3.062**	NM_023111
docking protein 1, 62kDa (downstream of tyrosine kinase 1)	DOK1	**-3.56**	NM_001381
lysyl oxidase	LOX	**-3.66**	NM_002317

Gene	Symbol	U87/MGMT vs U87/EV Fold Change	GenBank
Biological Processes			
Transmemebrane receptor protein tyrosine kinase signaling pathway (cont.)			
eukaryotic translation initiation factor 4A binding protein 2	EIF4EBP2	**-3.78**	AK001936
ninein (GSK3B interacting protein)	NIN	**-4.256**	NM_020921
vascular endothelial growth factor A	VEGFA	**-5.33**	NM_001025366
phospholipase C, epsilon 1	PLCE1	**-8.57**	NM_016341
fibronectin 1	FN1	**-12.53**	NM_212482
ephrin-B3	EFNB3	**-15.57**	NM_001406
vascular endothelial growth factor receptor 2	KDR	**-16.73**	NM_002253
ephrin-B1	EPHB1	**-19.31**	NM_004441
fibroblast growth factor 5	FGF5	**-20.2**	NM_004464
anaplastic lymphoma kinase (Ki-1)	ALK	**-76.26**	NM_004304
SHC (Src homology 2 domain containing) transforming protein 3	SHC3	**-103.95**	NM_016848
metastasis suppressor 1	MTSS1	**-172.2**	NM_014751
epiregulin	EREG	**-173.21**	NM_001432
necdin homolog (mouse)	NDN	**-210.9**	NM_002487
colony stimulating factor-1 receptor	CSF1R	**-222.46**	NM_005211

2.4.5. Differential expression of VEGFR-1 and -2 based on MGMT expression

Our gene expression profiling suggested that overexpression of MGMT induced major changes in the expression of several genes involved in angiogenesis, which has great significance for tumorigenicity and the response to antiangiogenic inhibitors. Thus, we selected VEGFR-1 and -2 for validation of their differential expression using measurements of RNA and/or protein in MGMT(+) and MGMT(-) cell lines. Quantitative real time RT-PCR (QRT-PCR) confirmed that *VEGFR-1* mRNA expression was increased in U87/MGMT and T98G cells compared to U87/EV cells. This increase was confirmed at the protein level by flow cytometry (Figure 2.6A). The decreased expression of *VEGFR-2* in U87/MGMT and T98G cells compared to U87/EV cells was also validated by QRT-PCR and flow cytometry (Figure 2.6B).

To investigate whether this differential expression was related to MGMT expression, U87/MGMT and T98G cells were treated with O6BG (20 μM, 48 h) to deplete MGMT, and the expression of VEGFR-1 and -2 was assessed by QRT-PCR and flow cytometry. As shown by immunoblotting, exposure to O6BG completely depleted MGMT protein in U87/MGMT and T98G cells (Figure 2.7A). Depletion of MGMT protein was associated with a significant decrease of *VEGFR-1* mRNA and protein expression in U87/MGMT ($P = 0.004$, Figure 2.7B) and T98G cells ($P = 0.0005$, Figure 2.7C). In contrast, treatment with O6BG significantly increased expression of *VEGFR-2* at the mRNA and protein level in U87/MGMT ($P = 0.002$, Figure 2.7B) and T98G cells ($P = 0.0007$, Figure 2.7C).

Our data support the association between MGMT levels and regulation of VEGFR-1 and -2 expression in GBM cells.

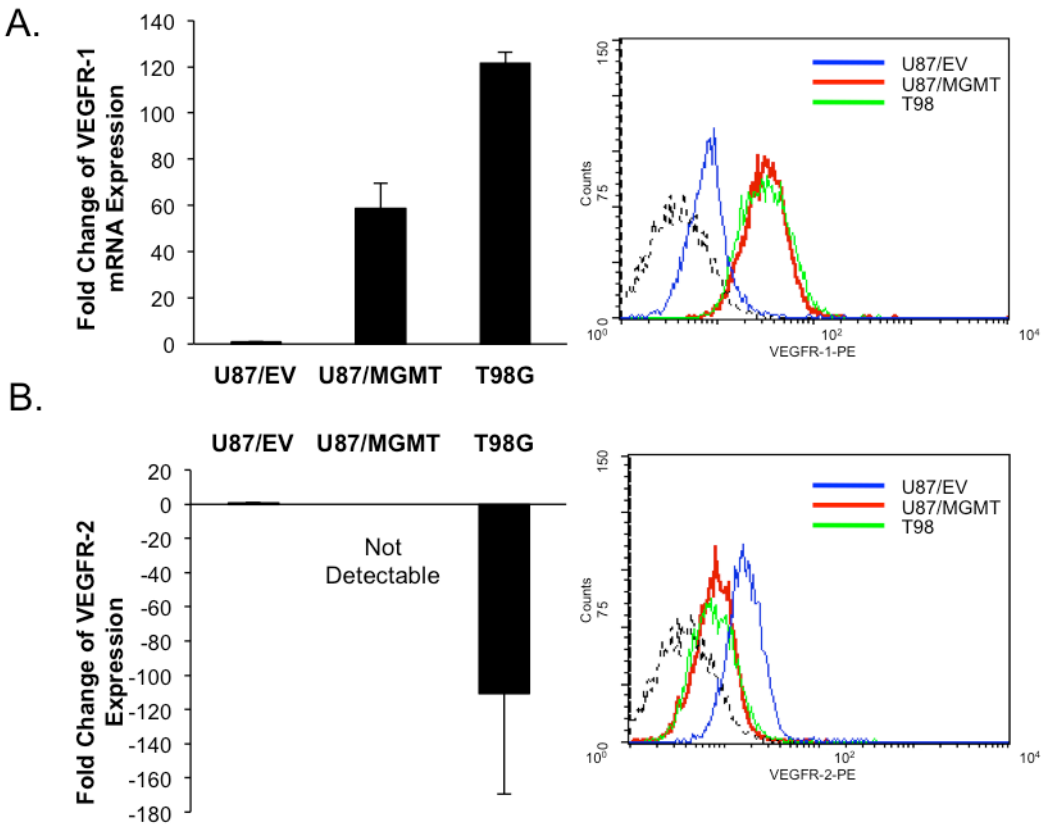

Figure 2.6. VEGFR-1 and -2 are differentially expressed in MGMT(+) cell lines. (A) Expression of VEGFR-1 by QRT-PCR (left panel) and flow cytometry (right panel) in various cell lines. **(B)** Expression of VEGFR-2 by QRT-PCR (left panel) and flow cytometry (right panel) in various cell lines. mRNA expression was analyzed using the comparative Ct method, and values are represented as fold change compared to U87/EV cells. Error bars represent the SEM of 3 independent experiments.

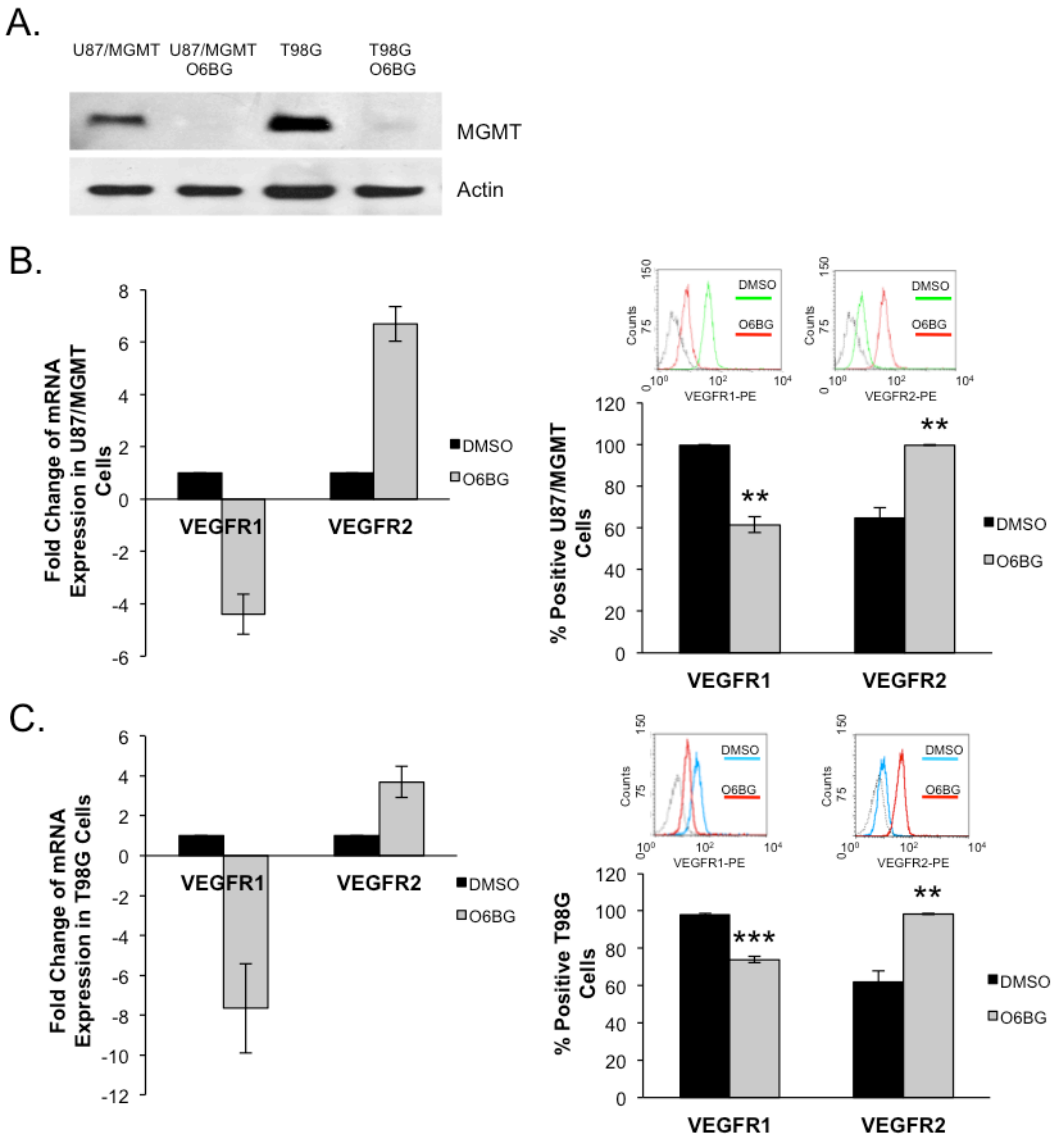

Figure 2.7. Expression of VEGFR-1 and -2 correlates with MGMT expression. **(A)** Expression of MGMT protein following treatment with O6BG (20 μM, 48 h) in U87/MGMT, and T98G cells. **(B)** Expression of VEGFR-1 and -2 by QRT-PCR and flow cytometry in U87/MGMT cells following treatment with O6BG. **(C)** Expression of VEGFR-1 and -2 by QRT-PCR and flow cytometry in T98G cells following treatment with O6BG. mRNA expression was analyzed using the comparative Ct method, and values are represented as fold change compared to DMSO control condition. Error bars represent the SEM of 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

2.4.6. Decreased secretion of VEGFA and sVEGFR-1 is accompanied by reduced angiogenic potential of MGMT(+) cell lines

VEGFA, a key angiogenic factor strongly expressed by tumor cells, is involved in the growth and malignant progression of GBM tumors, mostly through VEGFR-1 and VEGFR-2 (Plate, Breier et al. 1992; Kerbel 2008). According to our differential expression profile, expression of *VEGFA* was decreased in U87/MGMT cells (Table 2.2). ELISA analysis showed that U87/EV cells secreted significantly more VEGFA than U87/MGMT or T98G cells (P = 0.002 and 0.05, respectively) (Figure 2.8A), suggesting that regulation of the endogenous expression of VEGFA is related to MGMT expression in GBM cells.

sVEGFR-1, produced by alternative splicing, inhibits VEGFA signaling by sequestering the VEGF ligand (Kendall and Thomas 1993) and acts as a negative modulator for the bioactivity of VEGFA (Hornig and Weich 1999). Quantification of sVEGFR-1 by ELISA showed that this species was undetectable in U87/EV cultures but was secreted by U87/MGMT and T98G cells (Figure 2.8A). The marked increase in sVEGFR-1/VEGFA ratio in MGMT(+) cells (Figure 2.8B) would be expected to greatly decrease signaling through the VEGFR-1 and -2 receptors.

Next, to investigate the biological significance of differential secretion of VEGFA and sVEGFR-1 between U87/EV and U87/MGMT cells, we assessed angiogenesis using an *in vitro* assay. We tested how conditioned medium from these two cell lines influenced the ability of HMEC-1 endothelial cells to form tubular structures in matrigel (Figure 2.8C). Conditioned medium of U87/EV cells

induced a more extensive branching network with tube-like structures displaying multicentric junctions, compared to U87/MGMT-conditioned medium (mean tube length $P = 0.04$, mean tube area $P = 0.03$, number of nodes $P = 0.03$) (Figure 2.8C). Thus, secretion of angiogenic factors by MGMT(-) cells elicited a significantly greater *in vitro* angiogenic response than MGMT(+) cells.

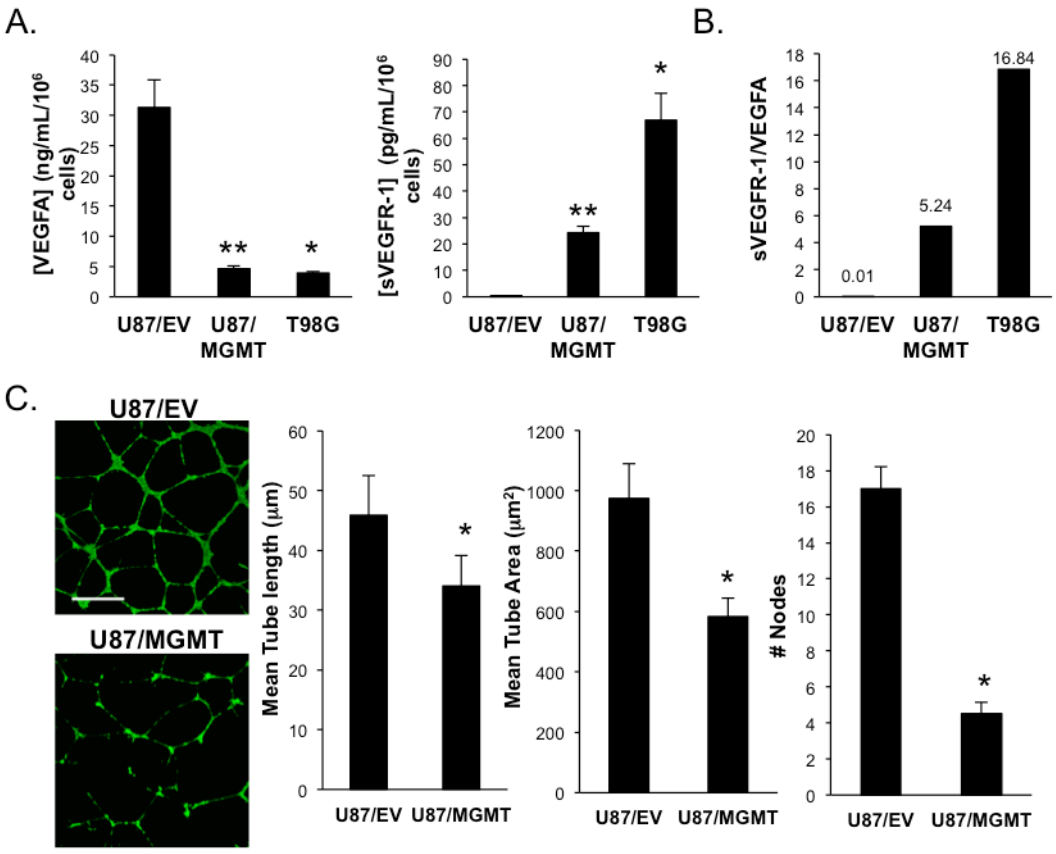

Figure 2.8. Regulation of angiogenic factors in MGMT(+) cells influences angiogenic potential. (A) Secretion of VEGFA and sVEGFR-1 as determined by ELISA assay. Error bars reflect the SEM of at least 3 independent experiments. **(B)** Ratio of sVEGFR-1 to VEGFA; a representation of the relative amount of bioactive VEGFA. **(C)** Representative photomicrographs (left) of the effect of conditioned medium (24 h) from U87/EV and U87/MGMT cells on HMEC-1 cell tube formation in matrigel. Cells were fluorescently stained with calcein AM. Scale bar = 500 μm. Tube formation was quantified (right) using MetaMorph 7.6 software, and error bars represent the SEM of 3 independent experiments. $*P < 0.05$, $**P < 0.01$.

2.5. DISCUSSION

Our study highlights for the first time the sensitivity of MGMT(+) versus MGMT(-) GBM cells to sunitinib. To understand how MGMT alters the expression of genes involved in the response to sunitinib, we performed a cDNA microarray study using an MGMT(-) GBM cell line and its MGMT(+) counterpart. Gene expression profiling revealed alterations in the angiogenic profile, as well as differential expression of several RTKs targeted by sunitinib. To our knowledge, our study is the first to suggest a relationship between MGMT expression and the angiogenic profile in human GBM. Notably, a large number of key positive regulators of GBM angiogenesis such as *VEGFA, VEGFR-2, neuropilin 2, colony stimulating factor (CSF3),* and *acidic fibroblast growth factor (FGF1)* (Roskoski 2007) were decreased in U87/MGMT cells, whereas other genes known for their anti-angiogenic activity such as *semaphorin 3F, endostatin,* and *COL4A1 (arrestin)* (Roskoski 2007) were increased. For gene validation, we selected genes/proteins that were directly involved in angiogenesis and are targets of sunitinib, namely VEGFR-1 and VEGFR-2 (Mendel, Laird et al. 2003). MGMT(+) cell lines (U87/MGMT and T98G) displayed higher levels of VEGFR-1 mRNA and protein levels compared to U87/EV cells, whereas they displayed decreased levels of VEGFR-2. More importantly, depleting MGMT using O6BG suggested that the expression of these receptors was directly related to MGMT levels.

The validation in MGMT(+) cells of decreased VEGFA, a primary mediator of angiogenesis, and of increased sVEGFR-1, which sequesters VEGFA

and thereby negatively regulates VEGF-mediated angiogenesis (Toi, Bando et al. 2002), has a great significance. Decreased sVEGFR-1/VEGFA ratio was previously shown to correlate with increased bioavailability of VEGFA and a pro-angiogenic phenotype in malignant GBM compared to diffuse astrocytoma (Lamszus, Ulbricht et al. 2003). In our study, we show that MGMT expression was associated with a dramatic increase of sVEGFR-1/VEGFA ratio, thereby suggesting a decrease of bioactive VEGFA, which shifts the balance in favor of an anti-angiogenic phenotype in MGMT(+) GBM cells. Several lines of evidence support the tenet that our MGMT(+) cells display an anti-angiogenic phenotype compared to the MGMT(-) GBM cell line: *(i)* their increased response to sunitinib-based therapy; *(ii)* the decreased activity of their conditioned media when tested in the *in vitro* tube formation assay; and *(iii)* their low tumorigenicity. In this regard, earlier studies showed that inhibition of VEGFA-induced angiogenesis (Kim, Li et al. 1993) and inhibition of endogenous expression of VEGFA suppressed tumor growth *in vivo*, thereby supporting the role of VEGFA as a major determinant of both angiogenicity and tumorigenicity of U87MG cells (Cheng, Huang et al. 1996). Thus, our study is the first to suggest a direct link between MGMT expression and decreased angiogenicity and tumorigenicity of GBM cells.

With respect to the large number of genes that were differentially expressed in U87/MGMT versus U87/EV cells, elucidating the intricate mechanism(s) by which MGMT induced these transcriptional alterations is challenging. We speculate that overexpression of MGMT altered expression of

these genes through indirect mechanisms and at different levels of regulation, including: *(i)* regulation of a transcription program that includes induction of a number of transcription factors, such as zinc finger domain proteins acting as activators or repressors of gene expression (Figure 2.5); or *(ii)* regulation through common epigenetic alterations in GBM, such as DNA hypermethylation or hypomethylation at the CpG island (CGI) promoters affecting genes that control cell growth, apoptosis, and angiogenesis (Nagarajan and Costello 2009). Of interest in this regard is the observation from our gene array data that expression of DNA methyltransferase 3a (*DNMT3a*) was significantly decreased in U87/MGMT cells, and this finding was validated by QRT-PCR (data not shown).

To date, MGMT has been described as a DNA repair protein which protects DNA from the mutagenic actions of endogenous carcinogens and elicits resistance to alkylating agents (Pegg, Dolan et al. 1995; Kaina, Christmann et al. 2007). Our study suggests a novel function(s) for MGMT as a negative upstream regulator of key functional pathways involved in angiogenesis and tumorigenicity. Further work using additional cell lines and archived surgical specimens from GBM patients is needed to decipher how MGMT mediates these functions and to validate the concept that MGMT expression shifts the angiogenic profile. Our *in vitro* and *in vivo* studies suggest that MGMT(-) GBM cells did not derive benefit from addition of sunitinib to standard therapy. The combination of sunitinib with the standard treatment may inhibit tumor growth of MGMT(+) cells by exerting not only direct anti-proliferative effects on tumor cells, but also anti-angiogenic effects through a concerted action on tumor cells expressing MGMT and the

tumor vasculature *in vivo*. We were unable to test this hypothesis in the current study because of the poor tumorigenicity of the MGMT(+) cell lines.

The validation of the relationship between MGMT expression and an angiogenic profile in GBM tumor samples may ultimately lead to prospective testing of MGMT expression before offering anti-angiogenic agents in combination with RT and TMZ in future clinical trials.

2.6. ACKNOWELDGEMENTS

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Chapter 3: O^6-methylguanine-DNA methyltransferase is a novel negative effector of invasion in glioblastoma multiforme

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3.1. SUMMARY

The dismal prognosis of glioblastoma multiforme (GBM) is mostly due to the high propensity of tumor cells to invade. We have previously reported an inverse relationship between GBM angiogenicity and expression of the DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT) known for mediating resistance to alkylating agents used in GBM treatment.

In the present study, given the crucial role of angiogenesis and invasion in GBM pathogenesis, we aimed to investigate the relationship between MGMT expression and GBM invasion. Stable overexpression of MGMT in the U87MG cell line significantly decreased invasion, altered expression of invasion-related genes, decreased expression of $\alpha_5\beta_1$ integrin and focal adhesion kinase, and reduced mesenchymal morphology and migration compared to the empty vector control. Conversely, shRNA-mediated stable knockdown of MGMT or its pharmacological depletion in the MGMT-positive T98G cell line were required for increased invasion. The inverse relationship between MGMT and invasion was further validated in primary GBM patient-derived cell lines. Using formalin-fixed paraffin-embedded tumors from patients diagnosed with primary GBM (n = 59), tumor *MGMT* promoter hypermethylation (*MGMT* gene silencing) was significantly associated with increased immunohistochemical expression of the pro-invasive matricellular protein SPARC ($P = 0.039$, chi-square test).

Taken together, our findings highlight for the first time the role of MGMT as a negative effector of GBM invasion. Future studies are warranted to elucidate the role of SPARC in the molecular mechanisms underlying the inverse relationship

between MGMT and GBM invasion and the potential use of MGMT and SPARC as biomarkers of GBM invasion.

3.2. INTRODUCTION

The lethality of glioblastoma multiforme (GBM) stems from its pronounced infiltrative potential, as cells can diffusely invade beyond the margin of therapeutic resection (Giese, Bjerkvig et al. 2003). Consequently, the prognosis of GBM remains poor, with a median survival of only 15 months following standard of care therapy involving surgery, radiotherapy, and chemotherapy with the alkylating agent temozolomide (TMZ) (Stupp, Mason et al. 2005). While the putative invasive process (i.e., detachment from the primary tumor site, receptor-mediated adhesion to the extracellular matrix (ECM), degradation of the ECM, and morphological alterations) is well characterized (Nakada, Okada et al. 2003), the mechanisms by which cells instigate this invasive behavior are still under scrutiny. Therapeutic advances for GBM require a detailed understanding of the primary mediators of this behavior, as regulators of invasion may play a role in determining patient survival (Rich, Hans et al. 2005). Though potential regulators such as the p75 neurotrophin receptor (Johnston, Lun et al. 2007), doublecortex, semaphorin 3B, secreted protein acidic and rich in cysteine (SPARC) (Rich, Hans et al. 2005), CCAAT-enhancer-binding protein β (C/EBPβ), and signal transducer and activator of transcription 3 (STAT3) (Carro, Lim et al. 2010) have been elucidated, their applicability as clinical biomarkers or anti-invasive therapeutic targets has not been conclusively determined.

Currently, there are 3 commonly used biomarkers for patients with brain tumors: isocitrate dehydrogenase 1 (*IDH1*) mutation, 1p/19q co-deletion, and most notably, promoter methylation of the DNA repair protein O^6-methylguanine-

DNA methyltransferase (*MGMT*) (Tabatabai, Stupp et al. 2010). Tumoral expression of MGMT is proposed to mediate resistance to TMZ-induced cytotoxicity via alkyl transfer at the O^6 position of guanine (Esteller, Garcia-Foncillas et al. 2000). Accordingly, correlative studies show that patients with tumors displaying *MGMT* promoter hypermethylation or low expression of MGMT protein [i.e., MGMT(-)] are more likely to benefit from TMZ treatment, compared to patients with tumors displaying unmethylated *MGMT*, or high MGMT expression [i.e., MGMT(+)] (Hegi, Diserens et al. 2005; Kreth, Thon et al. 2011). These studies suggest that MGMT status could be used as a predictive marker for response to alkylating agents.

The association between *MGMT* promoter methylation and a hypermutator phenotype was highlighted in a comprehensive genomic characterization of GBM tumors (Network 2008). These alterations could influence functional pathways dictating tumor phenotype, including invasive behavior. A potential relationship between MGMT status and tumor invasion was described in invasive Crooke's cell adenomas, which have decreased expression of MGMT compared to non-invasive ordinary-type adenomas of Cushing's disease (Takeshita, Inoshita et al. 2009), and in gastric carcinoma, in which *MGMT* promoter methylation was associated with lymph node invasion (Park, Han et al. 2001). In the context of GBM, Brandes et al. (Brandes, Tosoni et al. 2009) recently observed that following treatment, GBM tumors with *MGMT* promoter methylation recurred at more distal sites from the initial radiation field. Though these studies suggest a potential relationship between MGMT expression and invasion, thus far, the

effect of MGMT expression on GBM invasiveness has not been explicitly investigated.

We have previously characterized a novel inverse relationship between MGMT expression and GBM angiogenicity (Chahal, Xu et al. 2010). Tumor angiogenesis and invasion, the major hallmarks of GBM aggressiveness share interdependent molecular mechanisms of regulation (Vajkoczy, Goldbrunner et al. 1999; Eccles 2004; Onishi, Ichikawa et al. 2011). We investigated the relationship between expression of MGMT protein and GBM invasiveness using isogenic overexpression and knockdown, pharmacological depletion of MGMT in GBM cell lines, and validation in primary patient-derived GBM cell lines. Given the prominent role of the matricellular protein SPARC in GBM invasion *in vitro* and *in vivo* (Schultz, Lemke et al. 2002; Thomas, Alam et al. 2010), we further investigated the relationship between tumor *MGMT* promoter methylation, its immunohistochemical expression, and SPARC expression in primary surgical biopsies of patients with GBM.

3.3. MATERIALS AND METHODS

3.3.1. Cell culture and drug treatment

GBM cell lines U251, A172, U373, U138, LN18, T98G purchased from American Type Culture Collection (ATCC) were originally authenticated by the ATCC using DNA profiling of short tandem repeat loci. U87-MG empty vector (U87/EV) and its MGMT-transfected derivative U87/MGMT (Aghi, Rabkin et al. 2006) have been extensively characterized in our previous study (Chahal, Xu et al. 2010). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen) and were used following regeneration of vials from frozen stock cultures periodically (approximately every two months). All cell lines retained their initial phenotypic, biochemical and functional characteristics including expression of MGMT, sensitivity to TMZ, proliferation and invasion patterns throughout the study.

Patient-derived primary GBM cell lines were established from enzymatic dissociation of diagnostic biopsy tumors from patients diagnosed with GBM based on their clinical and pathology reports (Dr. Joan Turner, Cross Cancer Institute). In some cases, testing was performed by immunohistochemistry (IHC) to further confirm GBM identity (expression of glial fibrillary acidic protein but no βIII-tubulin or oligodendrocyte markers analysis was conducted by Dr. Kenneth Petruk, University of Alberta). Primary cell lines maintained in DMEM/F12 supplemented with 10% FCS and 1% glutamate (Invitrogen) were used at the following passages: 10 cell lines < passage 10, 4 cell lines < passage

18. All the above cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2. Testing for mycoplasma contamination was performed using DNA fluorochrome staining.

O^6-benzylguanine (O6BG, Sigma) was dissolved in dimethylsulfoxide (DMSO). For depletion of MGMT using O6BG, cells were treated daily for 6 days with 10 μM O6BG or DMSO as a control.

3.3.2. Microscopy, immunofluorescence staining, and morphometric analysis

Bright field images were taken on a Zeiss Axiovert 200M microscope attached to a Seniscam camera using a Zeiss Plan-NEOFLUAR 10x/0.3 or 5x/0.15 lens.

For immunofluorescence analysis, cells were grown on coverslips coated with 8 $\mu g/cm^3$ of collagen-I or poly-L lysine (PLL). Cells were then fixed in 3.7% formaldehyde, permeabilized with 0.1% TritonX-100, and stained for filamentous (F)-actin using Alexa fluor 555 phalloidin-TRITC (Invitrogen), or for focal adhesion sites using anti-FAK(pY397) (BD Biosciences) followed by incubation with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen). The nucleus was visualized with DAPI (Invitrogen). Images were collected on a Zeiss LSM 710/ConfoCor Observer.Z1 microscope with a Zeiss Plan-APOCHROMAT 40x/1.3 DIC oil immersion lens.

Morphology was analyzed using Integrated Morphometry Analysis in MetaMorph 7.7 imaging software. Cell area and shape factor (resemblance to a

circle) were assessed, and morphology was determined by dividing cell area by shape factor. High range values indicate a more mesenchymal morphology.

3.3.3. Western blot analysis

Western blotting was performed as described previously (Chahal, Xu et al. 2010). Cells were washed twice with phosphate-buffered saline (PBS) and lysed with RIPA buffer (20 nM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM NaPP, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin) (Schueneman, Himmelfarb et al. 2003). 30 μg of protein (BCA protein assay kit, Pierce) was separated by 12% SDS-PAGE under reducing conditions and transferred onto polyvinylidine difluoride membranes. Membranes were probed for MGMT (Clone 3.1) CD29/integrin β_1, FAK(pY397) (all from BD Biosciences), FAK (Upstate), integrin α_5 (Cell Signaling), or β-actin (Sigma-Aldrich). Subsequently, membranes were washed, and incubated with horseradish-peroxidase conjugated secondary antibody for 1 h. The protein expression was visualized using an enhanced chemiluminescent detection system (Amersham Biosciences AB). Densitometric analysis (Adobe Photoshop CS3) shows relative band intensities normalized to levels of β-actin.

3.3.4. Cell motility assay

Non-directional cell motility was analyzed by 2D time-lapse microscopy. Cells were seeded at 75,000 cells/well in 6-well plates coated with collagen-I (8 $\mu g/cm^3$) and allowed to adhere for 45 min before imaging. Differential interference contrast (DIC) images were acquired every minute over a 3 h period

in 2 representative fields using a Zeiss Axiovert 200M microscope attached to a CoolSnap camera with a Zeiss Plan-NEOFLUAR 10x/0.3 lens. Composite videos were then constructed and assessed using the Track Spot Over Time function of BitPlane Imaris x64 software.

3.3.5. Invasion assay

In vitro cell invasion was measured using BD BioCoat Matrigel invasion chambers (BD Biosciences; 8μm pores) following manufacturer's instructions. Cells (2.5×10^4) were serum-starved for 24 h, seeded in top chambers with DMEM containing 0.5% FCS, and allowed to invade towards a chemoattractant (DMEM + 10% FCS) for 24 h. DMSO- or O6BG-treated cells were subjected to invasion assays on day 6 in the presence of DMSO or O6BG for 24 h. Membranes were fixed with 3.7% formaldehyde and stained with 1% crystal violet. Invasive cells were visualized by bright-field microscopy using a Zeiss Axioskop2 Plus microscope attached to an Axiocam color camera with a Zeiss FLUAR 5x/0.25 lens and counted using MetaMorph 7.7 software.

3.3.6. Generation of short hairpin RNA constructs and stable MGMT shRNA transfection

MGMT 29mer shRNA (3 constructs) and control shRNAs were subcloned into the pGFP-V-RS vector (OriGene) at *EcoR*I and *Hind*III sites. The shRNA-empty vector plasmid contains a nontargeting sequence. The MGMT subclone containing the 29-mer shRNA sequence (only antisense strands indicated: 5′-

GGACAAGGATTGTGAAATGAAACGCACCA-3') was used for further experiments.

T98G cells were seeded into 6-well plates and transfected with LipofectAMINE 2000 (Invitrogen) following manufacturer's protocol. Stable MGMT shRNA clones were generated by puromycin selection (2 mg/mL for 2 weeks) and subclones were selected based on GFP-positivity in single cells. T98shC1.1 was derived from T98shC1 following further exposure to puromycin.

3.3.7. Clonogenic survival assays following temozolomide treatment

Temozolomide (TMZ) (Schering-Plough) was dissolved in DMSO. Clonogenic survival analysis was performed by plating cells at a density of 1.2 x 10^3 cells/10 cm dish for TMZ treatment, or 2 x 10^2 cells/10 cm dish for DMSO control conditions. Cells were allowed to adhere overnight, then exposed to TMZ (100 μM) for 3 h. Cells were then maintained in DMEM + 10% FCS + 1% P/S for 10-14 days, after which cells were stained with 1% crystal violet. Colonies with >50 cells were counted manually. Surviving fraction was calculated as follows: (colonies formed/total cells plated)/plating efficiency (as determined by DMSO control plates).

3.3.8. Patients Samples and MGMT Promoter Methylation

Tumor tissue paraffin blocks were collected for GBM patients diagnosed and centrally reviewed in a single institution. Ethical approval was obtained according to institutional guidelines (2006, Tom Baker Cancer Center, Calgary,

Alberta, Canada). Only patients with primary gliomas WHO grade IV were evaluated (n = 78). *MGMT* promoter methylation status in GBM tumors was characterized by methylation specific polymerase chain reaction (MSP) (Hegi, Diserens et al. 2005; Hamilton, Roldan et al. 2010).

3.3.9. Immunohistochemical staining for MGMT and SPARC

Tissue microarray (TMA) sections (3 cores) were immunohistochemically stained for MGMT or SPARC on BenchMark XT (Ventana Medical Systems) using the technical protocol XT ultraView DAB v3. Antigen retrieval for MGMT or SPARC used an extended CC2 protocol or standard CC1 protocol, respectively (Ventana Medical Systems). Incubation with anti-MGMT antibody (1:50 for 2 h, clone MT3.1; Millipore) or anti-SPARC (1:20,000 for 60 min; AON-5031, Hematologic Technologies) was followed by incubation with UltraView HRP-conjugated antibody. Antigen detection was performed using UltraView diaminobenzidine chromogen (Ventana Medical Systems). Primary antibody was omitted in the negative control. Endothelial cells served as positive internal control for MGMT and SPARC. Immunostaining was scored by a neuropathologist (MCG) blinded for *MGMT* methylation status. Sections were digitalized using an Aperio scanner scope XT.

3.3.10. Statistical analysis

Data are reported as mean +/- SEM and are representative of at least 3 independent experiments. Student's *t* test was used to compare between sets of

data for cell lines. Spearman's rho correlation coefficients (*r* values) were calculated with SAS software to determine correlations between MGMT expression, invasion, and integrin expression. Correlation is significant at the 0.05 level (2-tailed). The relationship between MGMT and SPARC was assessed using chi-square test. *P* values < 0.05 denote statistical significance.

3.4. RESULTS

3.4.1. MGMT(+) GBM cells are less invasive than MGMT(-) cells

Our previous study showed MGMT expression inversely correlated with angiogenesis (Chahal, Xu et al. 2010). Because angiogenesis and invasion are critical in glioma aggressiveness and share common molecular effectors (Vajkoczy, Goldbrunner et al. 1999; Eccles 2004), we sought to determine whether MGMT expression influences GBM invasive potential. Using the Gene Ontology (GO) Consortium (Ashburner, Ball et al. 2000) to analyze a previously conducted cDNA microarray, gene expression profiling of U87/EV and U87/MGMT cells revealed several functional pathways were differentially regulated (Chahal, Xu et al. 2010). Interestingly, biological processes involved in invasion were significantly differentially modulated (Table 3.1). To assess the relationship between MGMT and GBM invasiveness, we investigated *in vitro* invasion of a panel of 6 established cell lines, as well as the MGMT(-) cell line U87MG stably transfected with a vector encoding for MGMT (U87/MGMT) or the control empty vector (U87/EV) (Aghi, Rabkin et al. 2006; Chahal, Xu et al. 2010). Western blotting analysis of MGMT showed that MGMT protein expression was undetectable in U87/EV, A172, U251 cell lines and low in the cell line U373MG (Kanzawa, Germano et al. 2003) (10% expression) [i.e. MGMT(-)]. The MGMT(+) cell lines include U138MG, (intermediate level of MGMT, 40% expression), U87/MGMT, T98G and LN18, which exhibit comparable levels of MGMT protein (Figure 3.1A).

Using the Matrigel invasion assay, we established that MGMT(+) cell lines exhibited significantly less invasion compared to MGMT(-) cell lines ($P < 0.001$). Remarkably, the invasive potential of U87/MGMT was dramatically decreased compared to U87/EV cells ($P < 0.001$) (Figure 3.1B). Furthermore, correlation coefficient analysis showed a significant negative correlation ($r = -0.83$, $P = 0.011$) between MGMT expression and invasiveness of GBM cell lines.

β_1 integrin, a regulator of glioma invasion, interacts with ECM components of the perivascular basal lamina (D'Abaco and Kaye 2007) and mediates intracellular signaling pathways controlling cytoskeletal organization and cell movement. Western blotting analysis of β_1 integrin and one of its binding partners, the α_5 subunit revealed a different pattern of expression in MGMT(+) and (-) cells. Notably, compared to U87/EV, U87/MGMT cells displayed doublet bands reminiscent of a differential glycosylation process (Gu, Isaji et al. 2009). Interestingly, there was a consistent trend of low total expression levels for both β_1 and α_5 subunits in MGMT(+) cells (Figure 3.1C) and a negative correlation between expression of MGMT and α_5 ($r = -0.805$, $P = 0.016$) or β_1 integrin subunits ($r = -0.903$, $P = 0.002$). Hence, expression of MGMT is significantly negatively correlated with invasion of GBM cell lines, which is in accordance with the alteration of the pattern of $\alpha_5\beta_1$ integrin expression.

Table 3.1. Differential regulation of GO biological processes and KEGG pathways involved in invasion.

Biological Processes involved in invasion

Term	Count	% of gene list	P value
GO:0006928~cell motility	78	3.48%	1.15E-07
GO:0016477~cell migration	56	2.50%	2.52E-07
GO:0032989~cellular structure morphogenesis	70	3.12%	0.00348199
GO:0009611~response to wounding	61	2.72%	0.00533553
GO:0007155~cell adhesion	114	5.08%	2.27E-05

KEGG pathways involved in invasion

Term	Count	% of gene list	P Value
hsa04512~ECM-receptor interaction	19	0.85%	0.01790055
hsa04510~focal adhesion	36	1.60%	0.01496102

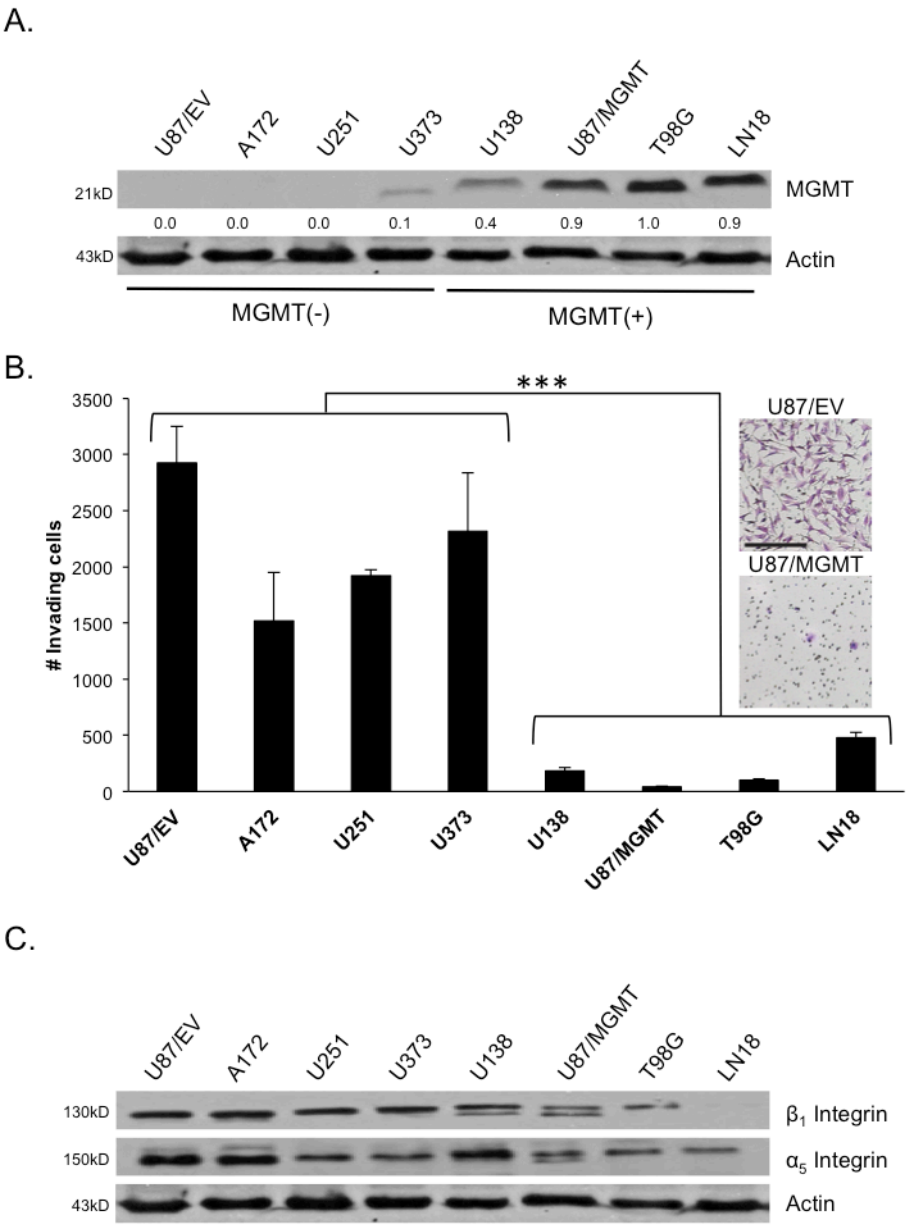

Figure 3.1. Inverse relationship between MGMT protein expression and invasion in human GBM cell lines. (A) Western blotting showing MGMT protein levels normalized to actin in 8 human GBM cell lines including U87MG cells stably transfected with empty vector (U87/EV) or MGMT (U87/MGMT). **(B)** Histogram shows the number of invading MGMT(-) compared with MGMT(+) cells assessed by Matrigel invasion assay (means ± SEM; n = 3); ***P < 0.001. Representative photomicrographs (inset) illustrate decreased invasion of U87/MGMT compared to U87/EV cells. Scale bar, 300 μm. **(C)** Immunoblots of the same panel of GBM cell lines shows expression of β_1 and α_5 integrin subunits. Note that overexpression of MGMT decreased invasion and reduced expression of $\alpha_5\beta_1$ integrin in U87/MGMT compared with U87/EV cells.

3.4.2. Overexpression of MGMT significantly decreased migration, mesenchymal morphology and expression of focal adhesion kinase

Next, we determined whether alterations in genes involved in migration (Table 3.1) translated to a functional modification of productive cell motility. We used 2D time-lapse video microscopy to assess non-directional cell migration on type I collagen (collagen-I), an ECM component found in perivascular regions of the brain (Gladson 1999) where GBM invasion occurs (Giese, Bjerkvig et al. 2003). Compared to U87/EV cells, we observed a stark contrast in the mode of migration of U87/MGMT cells. U87/EV cells move by extending elongated lamellipodia with a long and thin trailing edge, characteristic of the typical "stick-slip" pattern of GBM migration (Ulrich, de Juan Pardo et al. 2009). Conversely, U87/MGMT cells move by extending broad ruffled lamellipodia at the leading edge without a distinguishable trailing process (Supplementary movies S1 and S2, Figure 3.2A left panel). Compared to U87/EV cells, the mean migration speed of U87/MGMT cells was decreased by 29.7% ($P < 0.001$), and the mean displacement was strikingly decreased by 61.5% ($P < 0.001$) (Figure 3.2A right panel). Despite active formation of protrusions, overexpression of MGMT correlated with reduced productive movement and decreased the migratory speed, which may affect invasiveness of analyzed cells.

Since motility and invasion necessitate an alteration of morphology (Demuth and Berens 2004), we next assessed cytoskeletal F-actin organization of cells seeded on coverslips coated with PLL or collagen-I. As shown by rhodamine-labeled phalloidin staining, adhesion of U87/EV cells for 1 h induced

a mesenchymal morphology (Zhong, Paul et al. 2010) characterized by irregular lamellipodia and filopodia projections on PLL and increased spreading with evidence of clear actin stress fibers on collagen-I. In sharp contrast, U87/MGMT cells retained their round appearance regardless of substrate (Figure 3.2B, left panel). Additionally, quantitative evaluation of morphology revealed that U87/EV cells had a more mesenchymal appearance compared to U87/MGMT cells when plated on PLL ($P < 0.001$) or collagen-I ($P < 0.001$) (Figure 3.2B, right panel). After 24 h and 48 h U87/MGMT cells maintained a cobblestone-like morphology while U87/EV cells continued to display a more spindle-shaped, mesenchymal appearance on both PLL ($P < 0.001$ after 24 h and $P < 0.001$ after 48 h) and collagen-I ($P < 0.001$ after 24 h and $P = 0.007$ after 48 h). Notably, plating the cells on collagen-I compared to PLL did not induce significant morphological alterations of U87/MGMT cells at 24 h ($P = 0.77$), nor did it induce a change in morphology of either U87/EV or U87/MGMT cell lines at 48 h ($P = 0.45$ and $P = 0.59$ respectively) (Figure 3.3).

Focal adhesion kinase (FAK) is a primary mediator of the molecular link between ECM-bound integrins and the cell cytoskeleton, and thus is an important regulator of cell spreading, migration, and invasion (Natarajan, Hecker et al. 2003). GEP analysis revealed that the expression of the protein tyrosine kinase 2 gene (*PTK2)* encoding *FAK* mRNA was 2.25-fold lower in U87/MGMT cells ($P = 0.007$). Accordingly, immunoblotting showed that regardless of substrate, the expression of total FAK protein and FAK(pY397) were decreased in U87/MGMT compared to U87/EV cells at both 1 h and 24 h after plating (Figure 3.4A).

Furthermore, immunofluorescence staining of FAK(pY397) showed prominent focal adhesion sites along the elongated lamellipodia of U87/EV cells, but not in U87/MGMT cells plated on collagen-I (Figure 3.4B).

Thus, our results show that overexpression of MGMT correlated with profound morphological alterations, differential cytoskeletal F-actin reorganization and focal adhesion turnover, which may account for the reduced migratory and invasive phenotype in U87/MGMT compared to U87/EV cells.

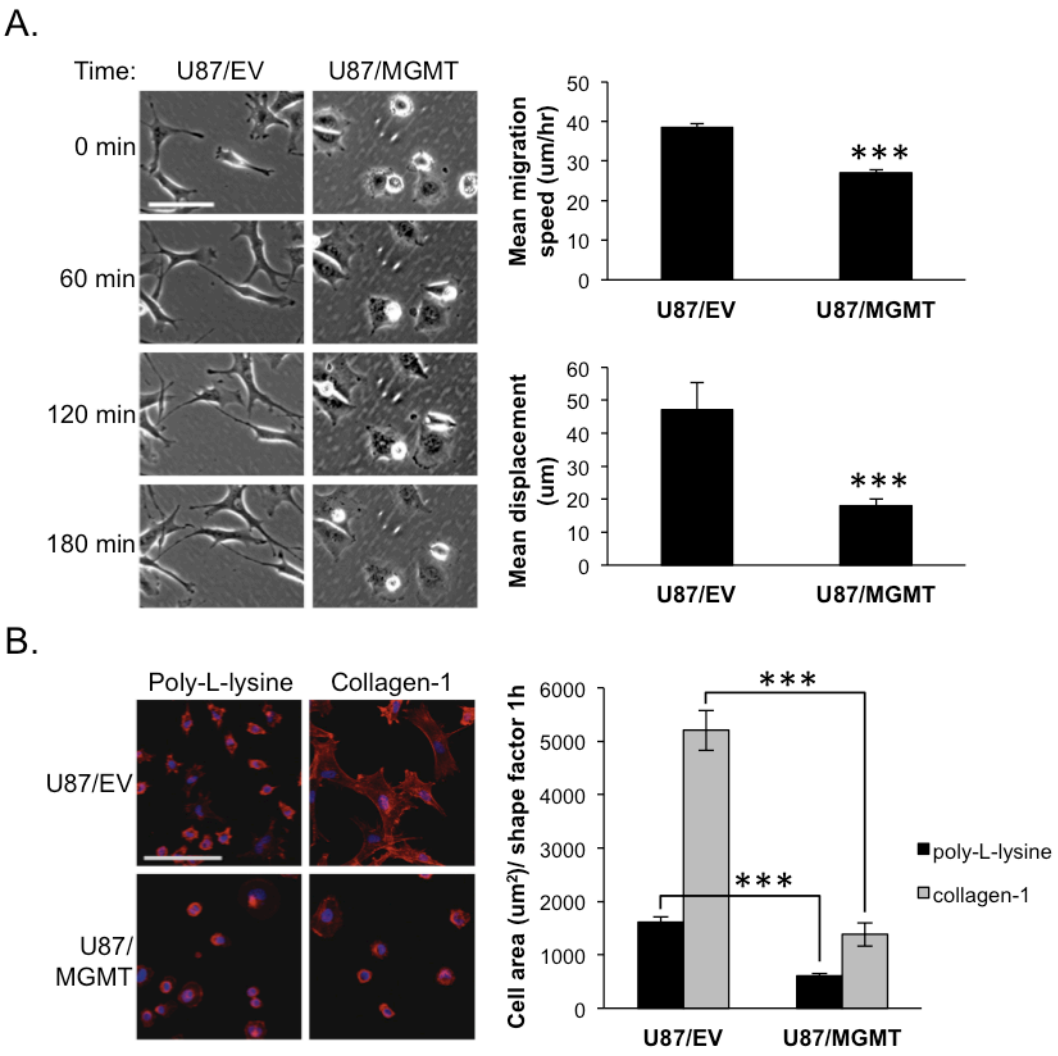

Figure 3.2. Overexpression of MGMT in U87MG cells decreased the migratory phenotype. (A) (*Left*) High-magnification images of U87/EV and U87/MGMT cells migrating on collagen-I and recorded by time-lapse video. (*Right*) Histograms show average migration speed and displacement in U87/EV and U87MGMT cells (> 50 cells). **(B)** (*Left)* U87/EV and U87/MGMT cells plated on collagen-I or poly-L-lysine for 1 h were stained for F-actin (red) and nuclear DNA (blue). (*Right)* Histogram shows quantitative evaluation of morphology as determined by cell area and shape factor (> 100 cells; means ± SEM; n = 3); ***$P < 0.001$. Scale bars, 100 μm.

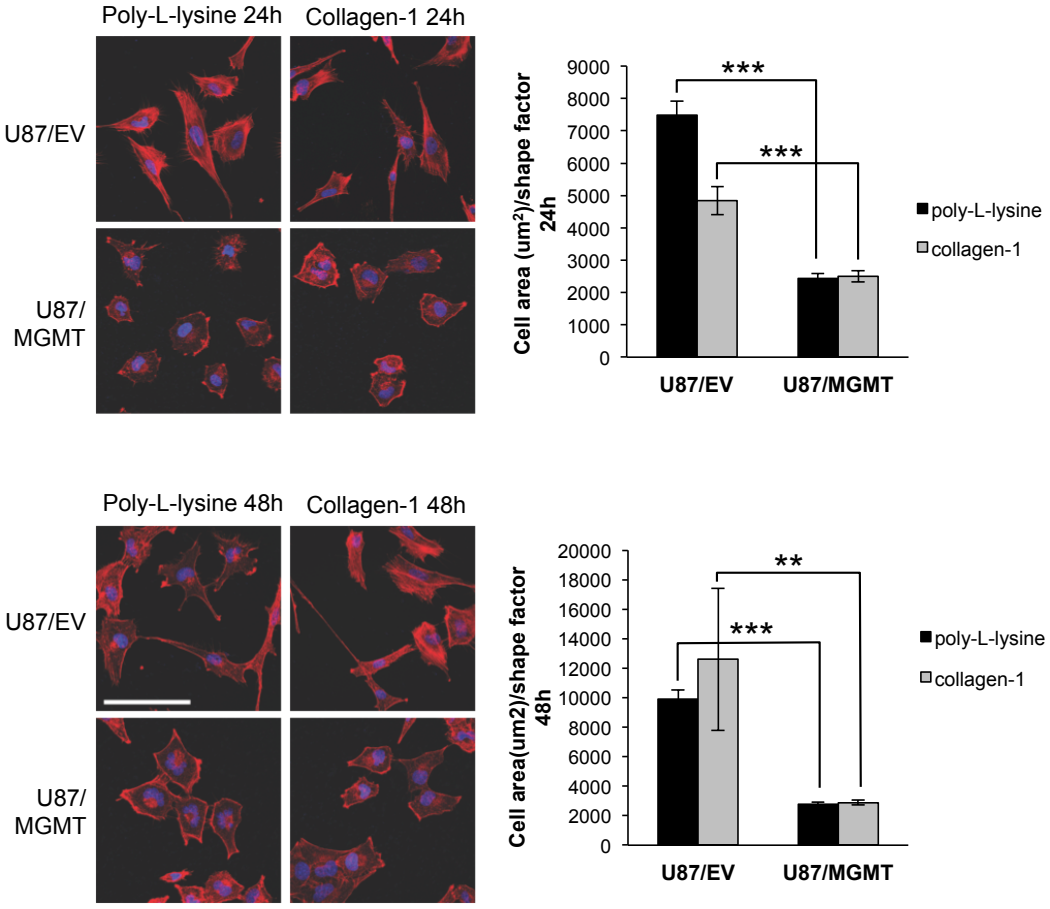

Figure 3.3. U87/MGMT cells have a less mesenchymal morphology than U87/EV cells after 24 and 48 h.

Substrate-independent alterations in cell morphology after 24 h and 48 h. U87/EV and U87/MGMT cells cultured on 8 μg/cm^3 collagen-I or poly-L-lysine coated coverslips were stained for F-actin (red) and nuclear DNA (blue); scale bar, 100 μm. Cell area and shape factor (right) was calculated using MetaMorph 7.7 software, and error bars represent the SEM of at least 100 cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

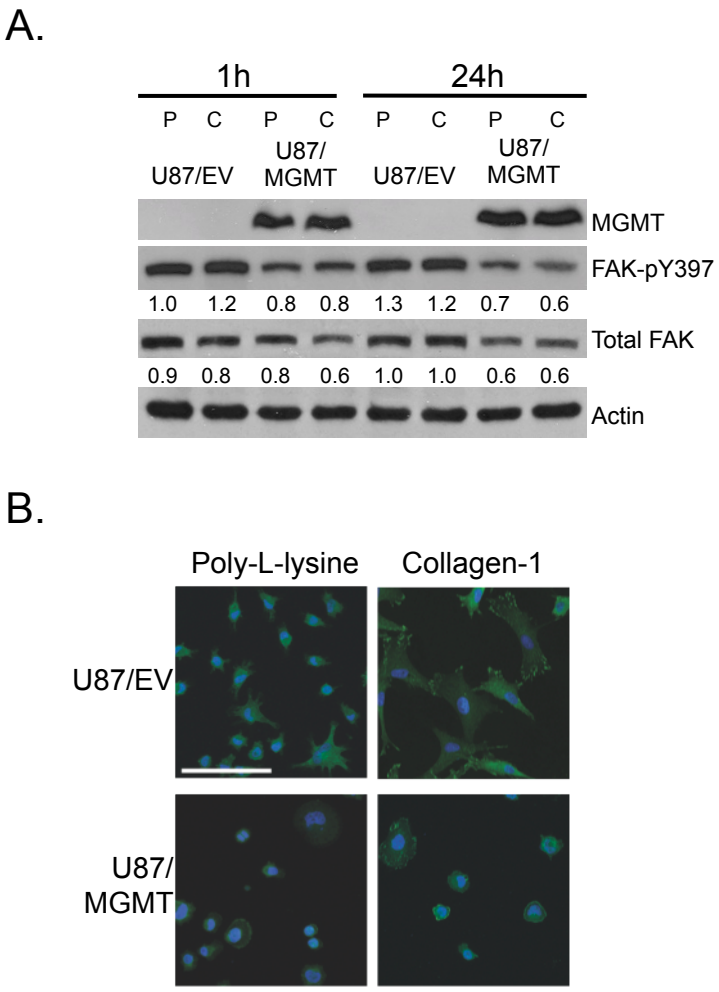

Figure 3.4. Overexpression of MGMT decreases expression and activation of FAK. (A) Western blotting showing FAK(Y397) and total-FAK levels normalized to actin in U87/EV and U87/MGMT cells plated on collagen-I or poly-L-lysine for 1 h and 24 h. **(B)** Immunofluorescence of FAK(Y397) (green) and nuclear DNA (blue) in U87/EV and U87/MGMT cells plated on collagen-I or poly-L-lysine for 1 h. Note that overexpression of MGMT (U87/MGMT) decreased migration, the mesenchymal appearance, activation and expression of FAK and the number of focal adhesion sites compared with U87/EV cells. Scale bars, 100 μm.

3.4.3. Depletion of MGMT is associated with increased GBM invasiveness

To establish proof-of-concept that MGMT expression influences invasion in GBM cells, we used stable shRNA-mediated knockdown of endogenous MGMT in T98G cells. Western blot analysis showed similar levels of MGMT protein in T98G cells stably transfected with empty vector (T98/EV) compared to parental cells, while MGMT was decreased in 2 randomly selected clones T98shC1 and T98shC8 (by 70% and 60%, respectively). Further knockdown of MGMT was induced in T98shC1.1, a sub-clone derived from T98shC1 (by 90%; Figure 3.5A). Importantly, MGMT knockdown functionally increased sensitivity to TMZ treatment in clonogenic survival assays, as only 34% of T98shC1 cell clones and 33% of T98shC1.1 cell clones survived (P = 0.002, P = 0.004, respectively), while T98/EV cell lines were almost completely resistant to TMZ treatment (87% survival, P = 0.25) (Figure 3.5B).

Remarkably, T98shC1, T98shC8 and T98shC1.1 clones displayed a more spindle-shaped morphology compared to the cobblestone-like morphology of T98G and T98/EV cell lines (Figure 3.6A). These phenotypic alterations translated into increased invasiveness only in the T98shC1.1 cell line (compared to T98/EV, P = 0.046, P = 0.008 compared to T98G) (Figure 3.6B).

The stark difference between invasion of either U87/MGMT and U87/EV (Figure 3.1B) or T98/EV and T98shC1.1 cell lines (Figure 3.6B), which had no detectable levels of MGMT protein, suggests that MGMT shRNA-induced knockdown in T98shC1 and T98shC8 cell lines was not sufficient to induce a shift in their invasion. Therefore, we further depleted MGMT using O^6-

benzylguanine (O6BG), a pseudosubstrate of MGMT that induces its degradation (Dolan, Moschel et al. 1990). Compared to their respective DMSO control conditions, treatment with O6BG (10 μM for 6 days) decreased MGMT expression by 80% in both T98G and T98/EV cells (Figure 3.7A). This depletion was associated with acquisition of spindle-shaped morphology in both cell lines (Figure 3.7B). When compared to DMSO control, treatment with O6BG further depleted MGMT protein by 20% and 30% in T98shC1 and T98shC8 cell lines respectively, thereby depleting the total amount of MGMT protein by 80% (Figure 3.7A). Remarkably, while O6BG treatment did not affect invasion of U87/EV cell line in the absence of endogenous MGMT, invasion was significantly increased by a fold change of 2.72 for T98G cells ($P = 0.027$), 2.07 for T98/EV cells ($P = 0.026$), 3.63 for T98shC1 cells ($P = 0.017$), and 2.06 for T98shC8 cells ($P = 0.019$) treated with O6BG compared to respective DMSO controls (Figure 3.7C). Thus, as demonstrated by shRNA-mediated knockdown of MGMT (T98shC1.1) and depletion by O6BG treatment, decreased MGMT expression by 80% seems to be a prerequisite for increased *in vitro* invasiveness of GBM cell lines.

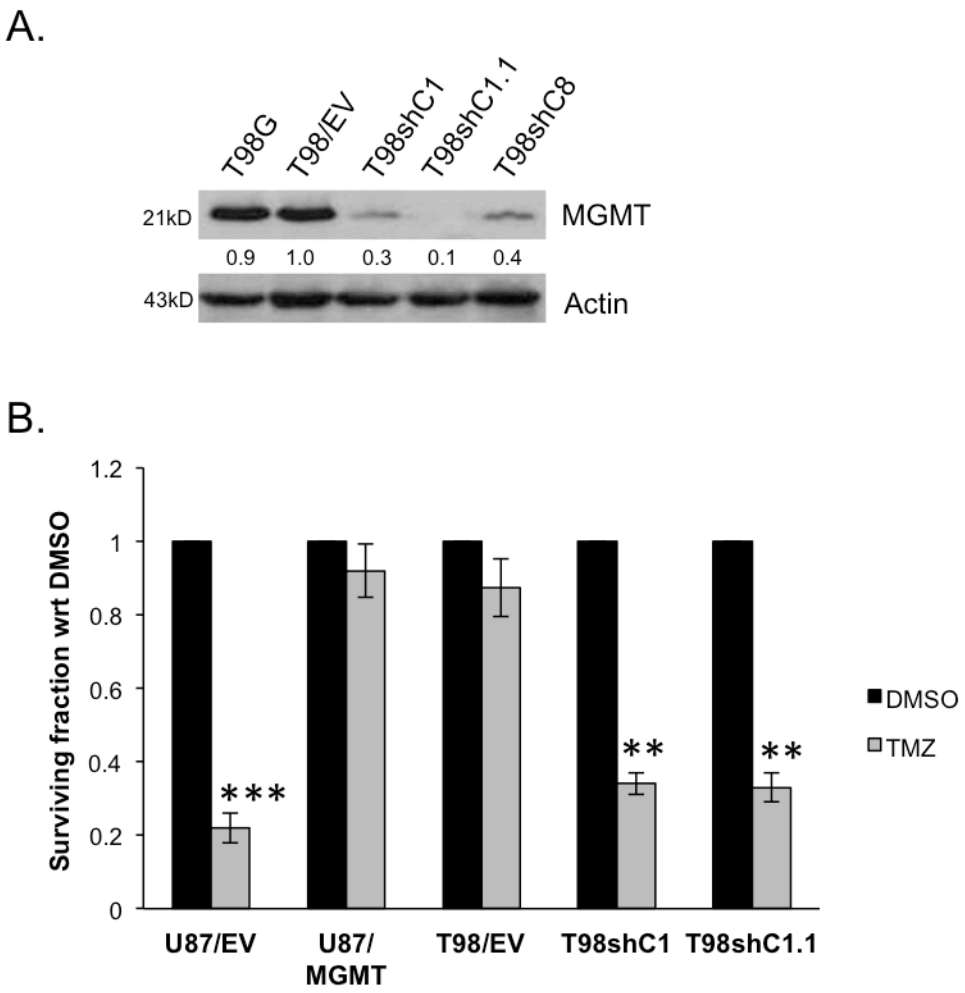

Figure 3.5. MGMT knockdown in T98G cells is associated with increased sensitivity to TMZ. (A) Western blotting of MGMT normalized to actin in T98G, T98/EV, T98shC1, T98shC1.1, and T98shC8 cells. **(B)** Histogram shows that MGMT overexpression in U87/MGMT cell lines rendered cells resistant to TMZ treatment, while MGMT-knockdown significantly increased sensitivity to TMZ treatment in T98shC1 and T98shC1.1 cell lines (means ± SEM; n = 3); $^{**}P < 0.01$, $^{***}P < 0.001$.

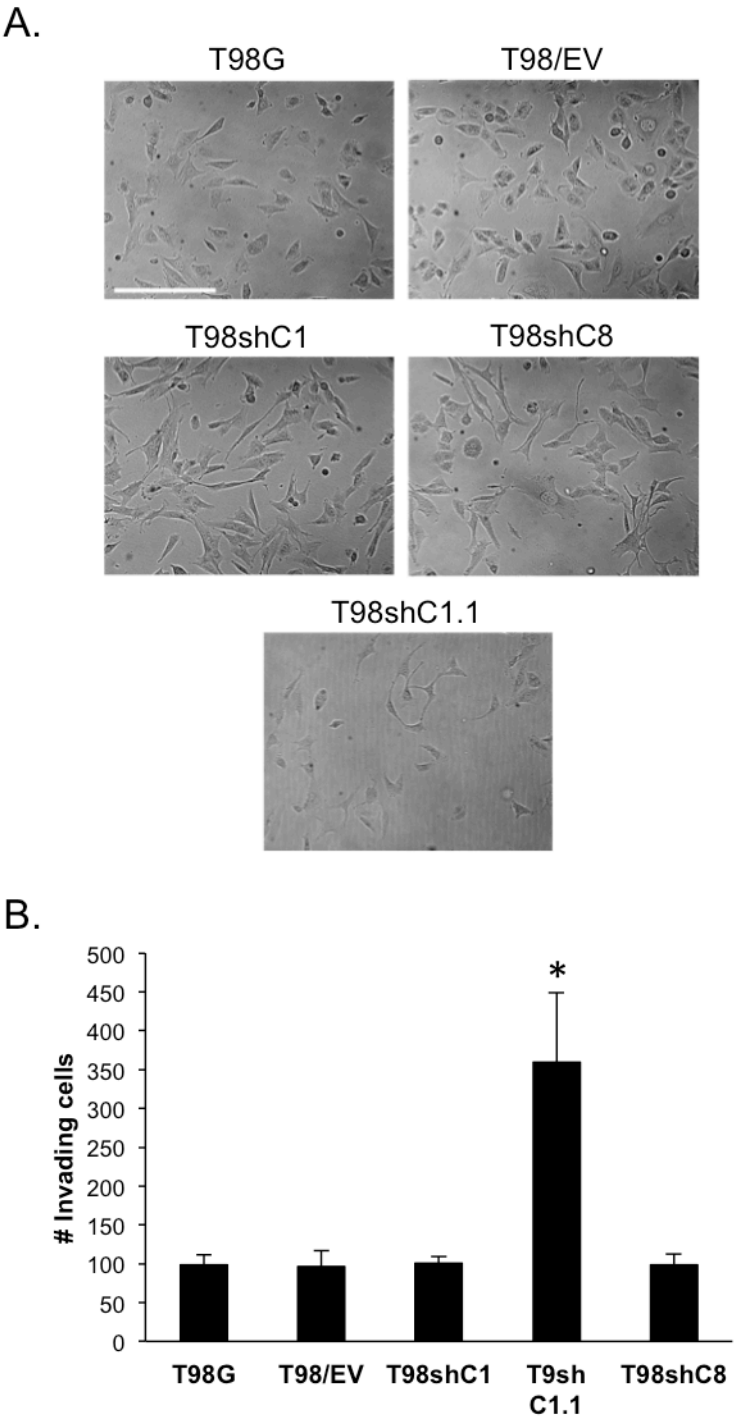

Figure 3.6. MGMT knockdown in T98G cells is associated with altered morphology and increased invasion. (A) Representative photomicrographs showing differential morphology of T98G cells, T98/EV cells, and three MGMT-knockdown clones T98shC1, T98shC8, and T98shC1.1; scale bar, 300 μm. **(B)** Histogram shows that MGMT-knockdown significantly increased invasion only in T98shC1.1 cells, as determined by Matrigel invasion assay (means ± SEM; n = 3); $*P < 0.05$.

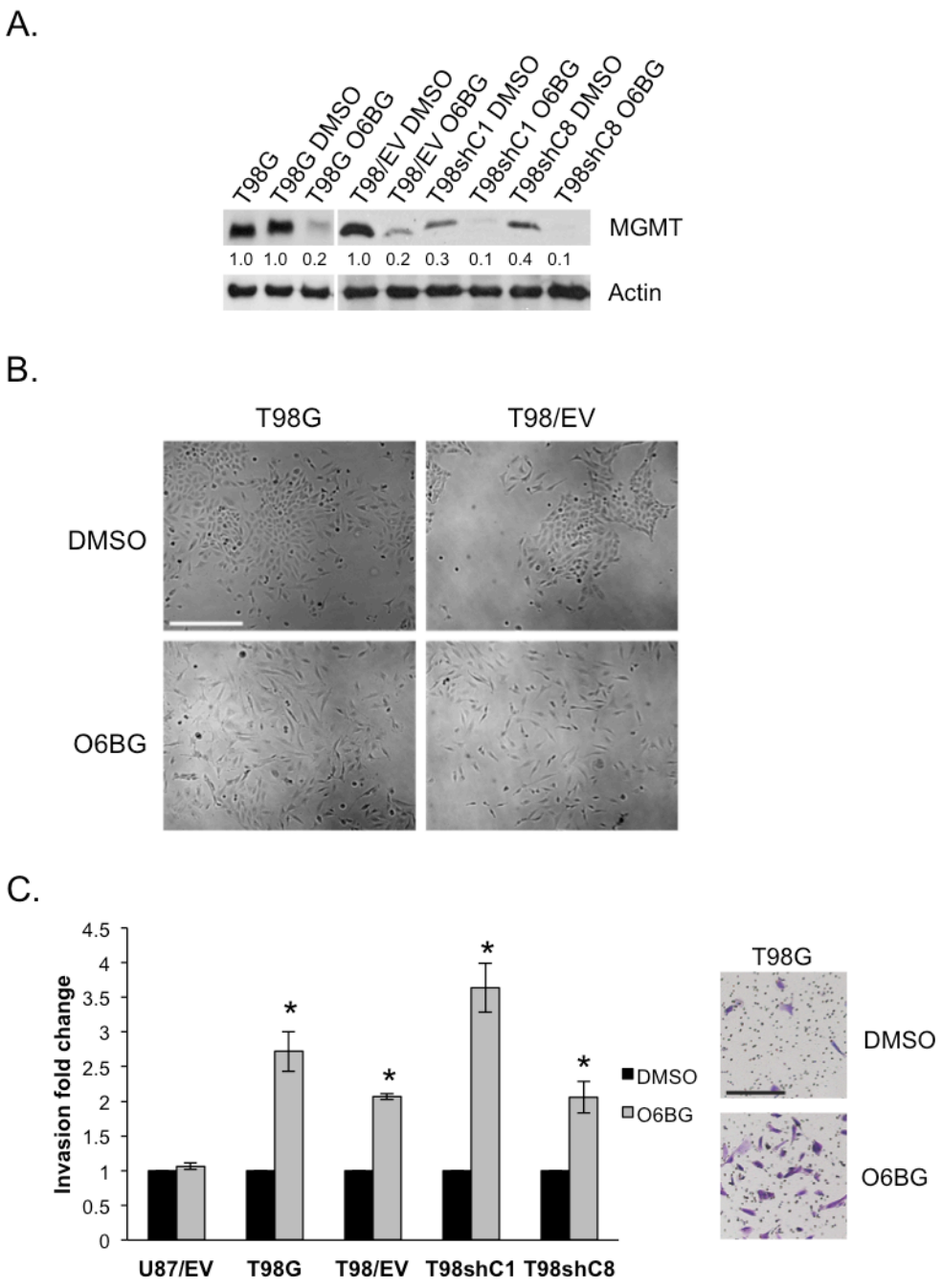

Figure 3.7. MGMT depletion by O6BG increases invasion of T98G cells. (A) Western blotting shows depletion of MGMT protein by O6BG (10 μM, 6 days) compared to DMSO control. **(B)** T98G and T98/EV cells treated with DMSO or O6BG (10 μM, 7 days); scale bar, 500 μm. **(C)** Histogram shows O6BG does not alter MGMT(-) U87/EV cell invasion, but increased invasion of T98G and T98/EV cells, as illustrated in representative photomicrographs (*right*, scale bar, 300 μm). Additional depletion of MGMT by O6BG is required for increased invasiveness of T98shC1 and T98shC8 cells (means ± SEM fold increase; n = 3; *, $P < 0.05$).

3.4.4. MGMT(+) primary patient-derived GBM cells display an invasive phenotype compared to MGMT(-) cells

To validate our findings in isogenic overexpression and knockdown models and determine their potential clinical validity, we examined invasion of 14 primary patient-derived GBM cell lines. Western blotting revealed that 9 out of 14 cell lines were MGMT(+) while the remaining 5 cell lines were MGMT(-) (Figure 3.8A). Overall, the MGMT(-) cell lines were significantly more invasive compared to the MGMT(+) cell lines ($P = 0.048$), and notably, 3 of the 5 MGMT(-) cell lines displayed levels of invasion comparable to U87/EV. Interestingly, 6 of the 9 MGMT(+) cell lines exhibited a similar invasive profile as U87/MGMT, while the remaining 3 were intermediately invasive (Figure 3.8B).

We further investigated whether depletion of MGMT protein was associated with increased invasiveness in primary cells. In the absence of endogenous MGMT protein, O6BG treatment of P-GM1 cells (10 μM for 6 days) did not significantly alter invasion. Depletion of MGMT by O6BG by 80% in P-GM7 and in P-GM13 cells was associated with a significant increase in invasion compared to DMSO control (1.85-fold, $P = 0.018$ and 2.46-fold, $P < 0.001$), respectively). Conversely, depletion of MGMT by only 60% in O6BG-treated P-GM5 cells (Figure 3.8C) did not induce a significant increase in invasion (Figure 3.8D). These findings corroborate that drastic decrease of MGMT expression is associated with increased *in vitro* invasion of established and primary patient-derived GBM cell lines.

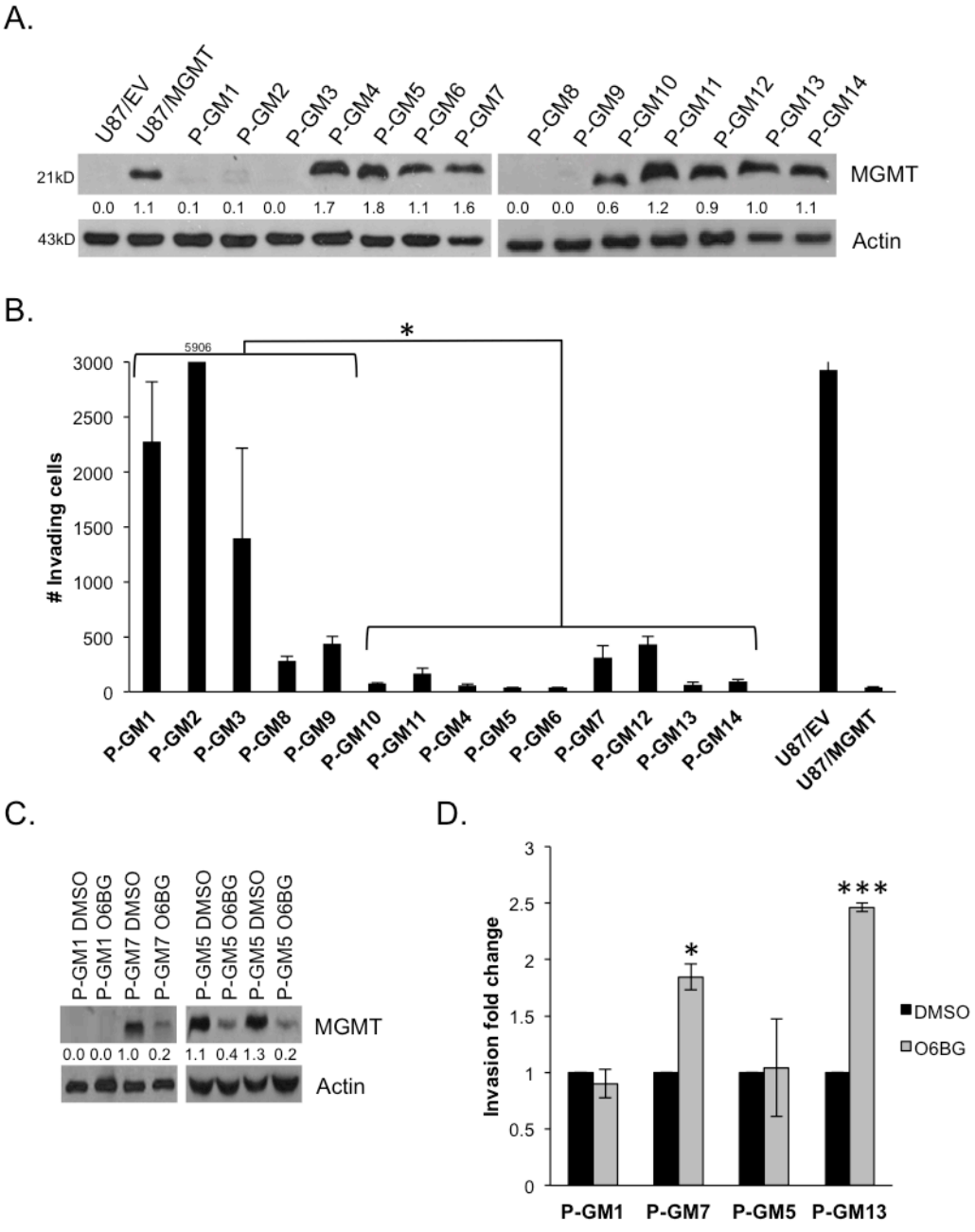

Figure 3.8. MGMT(+) primary patient-derived GBM cells are less invasive than MGMT(-) cells. (A) Western blotting shows MGMT expression normalized to actin in 14 primary GBM cell lines. **(B)** Histogram shows the number of invading MGMT(-) compared with MGMT(+) cells assessed by Matrigel invasion assay (means ± SEM; n = 3; **P* < 0.05). **(C)** Western blotting shows depletion of MGMT protein by O6BG (10 μM, 6 days) was dramatic in P-GM7 and P-GM13, but not in P-GM5 cells. **(D)** Histogram shows O6BG significantly increased invasion of P-GM7 and P-GM13, but not P-GM5 cells, and has no effect on MGMT(-) P-GM1 cell invasion (means ± SEM fold increase; n = 3; ***, *P* < 0.001).

3.4.5. Relationship between MGMT status and expression of SPARC in GBM patients

To substantiate the clinical relevance of our findings we sought to investigate the relationship between MGMT and the expression of SPARC, a well-known pro-invasive molecule (Arnold and Brekken 2009) in a series of tumor biopsies from newly diagnosed GBM patients with no prior history of radiotherapy or chemotherapy (n = 78). Because the clinical value of immunohistochemical detection of MGMT protein is still controversial, we first investigated the correlation between MGMT expression and SPARC using analysis of *MGMT* promoter methylation by MSP prospectively carried out in FFPE tumors from this cohort of patients. MSP results were not available in a total of 14 cases (insufficient tissue for analysis or technically unable to obtain results). Patients were dichotomized as methylated or unmethylated. *MGMT* promoter was found methylated in 58% (37/64) of cases, in accordance with previous reports (34%-68%, with a mean of 46%, (Weller, Stupp et al. 2010)).

For the purpose of this study, we assessed immunohistochemical expression of MGMT using TMA sections of the same series of GBM patients (n = 78). Twenty-five out of 78 cases (32%) were scored negative (0) or showed a heterogeneous tumor population (1+ and 2+), whereas 53 tumor samples (68%) showed homogeneous nuclear MGMT immunostaining (≥ 90% MGMT-positive tumor cells: 3+) (Figure 3.9A-F).

As previously reported, we observed a concordance between MGMT expression and *MGMT* promoter methylation status in 56% of analyzed samples

(Cao, Jung et al. 2009), while the subgroup of patients with unmethylated *MGMT* promoter displayed a stronger concordance with MGMT-immunopositivity (74%).

IHC staining of SPARC showed cytoplasmic localization in GBM tumor cells. Based on the percentage of cytoplasmic positive tumor cells, of the 70 GBM cases with available data (insufficient tissue on TMA sections, n = 8), 9 were scored as negative to weakly positive (13%, score = 0 or 1), 17 were moderately positive (24%, score = 2), and 44 were strongly positive (63%, score = 3) (Figure 3.9G-N).

Strikingly, *MGMT* promoter methylation was significantly associated with increased immunohistochemical expression of SPARC (score = 3 versus score = 0, 1 and 2) (n = 59 cases with available data for both MGMT status and SPARC by IHC). Up to 76% of cases (25 out of 33) with *MGMT* promoter methylation were strongly positive for SPARC ($P = 0.039$). In contrast, immunopositivity of MGMT failed to correlate with SPARC expression by IHC staining ($P = 0.405$).

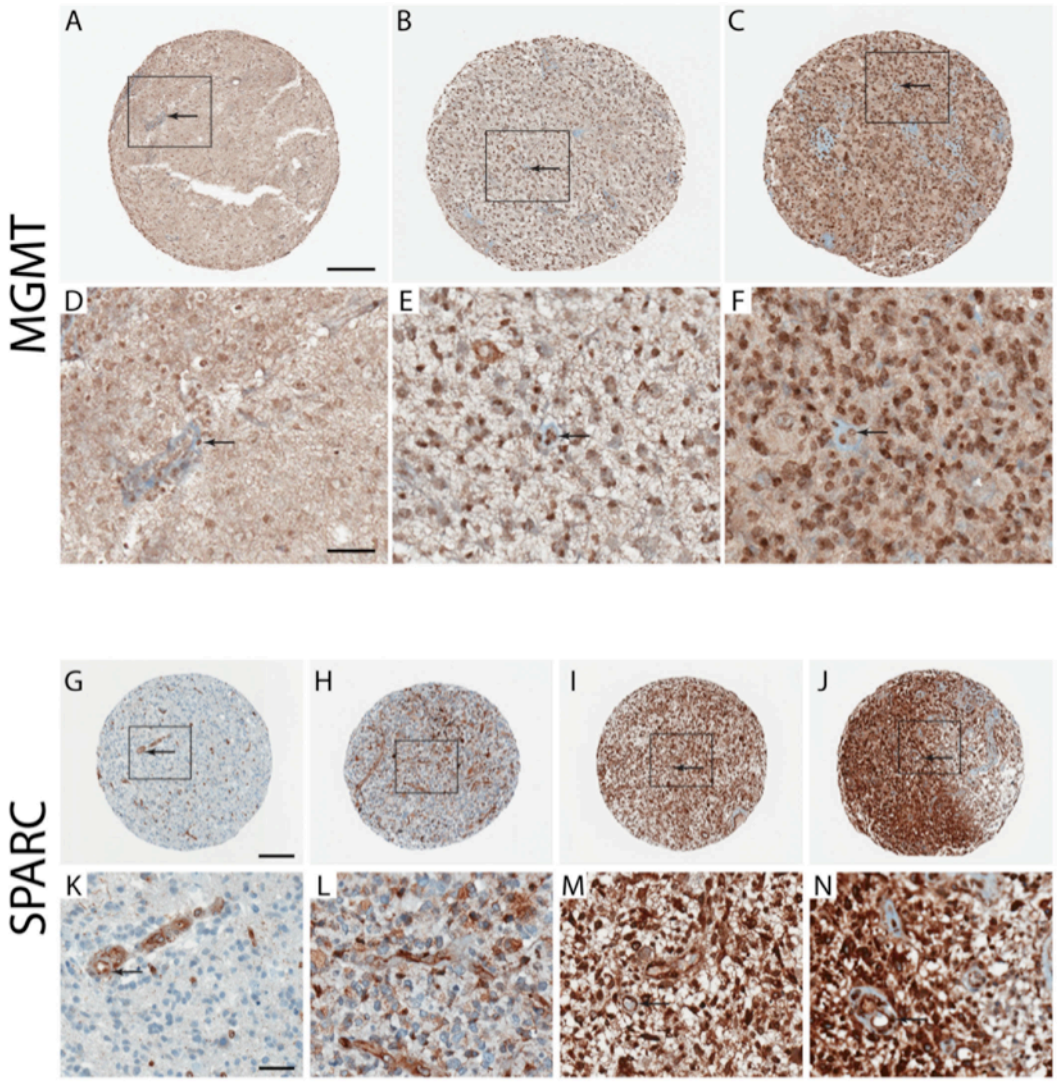

Figure 3.9. Representative immunohistochemistry staining of MGMT and SPARC. Photomicrographs display expression of MGMT (**A-F**; score: 0, 2+ and 3+, respectively) and SPARC (**G-N**; score: 0, 1, 2 and 3, respectively) in TMA of GBM patients (**A-C** and **G-J**). The insets show higher magnification (**D-F** and **K-N**). Endothelial cells were used as internal positive control (arrows). Scale bars, 200 μm (**A-C** and **G-J**), 50 μm (**D-F** and **K-N**).

3.5. DISCUSSION

In the present study, we identified a novel role for MGMT protein as a potential negative molecular and phenotypic regulator of GBM invasion, the main cause of treatment failure for patients with GBM. Using established and isogenic GBM cell lines differing in MGMT protein expression, primary GBM cell lines, and archived patient tumors, we provide the first direct evidence of an inverse relationship between MGMT expression and GBM invasiveness.

In particular, stable overexpression of MGMT decreased invasiveness of U87/MGMT cells compared to their counterpart and induced profound alterations of genetic, molecular and phenotypic features. First, GEP analysis revealed modulation of a plethora of key genes encoding structural and signaling proteins involved in cytoskeleton remodeling, cell adhesion and movement. Second, molecular determinants which may account for decreased invasion could be related to *(i)* a differential integrin profile, i.e., decreased total expression levels and presumably alterations of the maturation process of $\alpha_5\beta_1$ integrin, which may reduce cell spreading and migration (Gu, Isaji et al. 2009) and *(ii)* decreased expression and activation of FAK. Owing to the role of FAK in cell motility, we selected FAK for in-depth quantitative studies and validated decreased total expression of FAK shown by GEP analysis. In accordance with decreased Tyr397-FAK, immunofluorescence showed a reduced number of focal adhesion sites in U87/MGMT. Third, morphological changes were evidenced by *(i)* a differential cytoskeletal F-actin reorganization and cell spreading on PLL and collagen-I substrates *(ii)* a mesenchymal mode of migration for U87 cells

(Yamazaki, Kurisu et al. 2009), while quantitative morphometric analysis and monitoring cell migration by time-lapse evoked a switch from mesenchymal to an amoeboid motility for U87/MGMT cells. Failure to extend long polarized pseudopodia and decreased tyrosine phosphorylation of FAK in U87/MGMT agrees with studies showing the requirement for regulated focal adhesion turnover for mesenchymal motility (Carragher, Walker et al. 2006). Understanding how MGMT affects the dynamics of actin cytoskeleton turnover and elicits a mesenchymal–amoeboid transition deserves further validation on 3D substrates and may ultimately identify new targets to efficiently reduce invasiveness *in vivo*.

Overall, forced expression of MGMT provided some mechanistic insights into potential concerted effectors leading to decreased invasiveness. Conversely, shRNA-mediated knockdown of endogenous MGMT by 90% was significantly associated with increased invasion in T98shC1.1 cells. Interestingly, clonogenic assay of T98shC1 and T98shC1.1 cells indicated that though these cell lines displayed altered invasiveness, they responded similarly to TMZ treatment, suggesting that MGMT regulation of invasion may be independent of its alkyltransferase activity. Additionally, drastic depletion of MGMT by O6BG in T98G cells, as well as further depletion by O6BG in T98shC1 and T98shC8 cell lines was required to increase invasiveness compared to parental untreated cells.

While we establish for the first time the relevance of MGMT to invasion *in vitro*, validation of our findings in primary GBM cells and patients archived tumors highlights their potential clinical significance. We showed for the first time an inverse relationship between MGMT and the pro-invasive protein SPARC

in a series of primary GBM patients. Interestingly, SPARC has been shown to promote migration and invasion through direct physical interactions with β_1 integrin (Nie, Chang et al. 2008; Weaver, Workman et al. 2008) and activation of important signaling molecules for glioma cell motility, such as integrin-linked kinase and FAK (Barker, Baneyx et al. 2005; Shi, Bao et al. 2007)**.** In particular, *MGMT* promoter hypermethylation (which is expected to reflect low levels of MGMT protein) was significantly associated with high levels of tumoral SPARC expression. As previously reported in other studies, we found a limited concordance between immunopositivity of MGMT and promoter methylation (Preusser, Charles Janzer et al. 2008) despite our caution in analyzing immunohistochemical expression of MGMT in patients with similar high glioma grade and without prior treatment with chemotherapy or radiotherapy (Capper, Mittelbronn et al. 2008). Potential limiting factors for the validation of immunohistochemical analysis of MGMT as a marker of GBM invasiveness could be related to the relatively small sample size in our cohort and MGMT intratumoral heterogeneity. In particular, MGMT protein expression has been shown to decrease progressively from the inner to the peripheral layer in GBM samples (Della Puppa, Persano et al. 2011). Interestingly, tumor cells located in the brain parenchyma (Hoelzinger, Mariani et al. 2005) or beyond the margin of tumor resection (residual tumor cells) (Glas, Rath et al. 2010) are more invasive than cells within the tumor core.

Our study reveals that SPARC might be involved in the molecular mechanisms underlying the inverse relationship between MGMT and GBM

invasion. In a recent study, high expression of the membrane–cytoskeleton linker protein, ezrin was correlated with loss of MGMT expression and increased invasion in esophageal cancer (Su, Liu et al. 2011). Physical interactions of MGMT with binding partners may also account for downstream alterations of biological processes, such as the transcription integrator CREB-binding protein CBP/p300 (Teo, Oh et al. 2001) and the Histone acetyltransferase p300 (EP300) known to regulate transcription via chromatin remodeling [Protein Interaction Network Analysis: PINA (Wu, Vallenius et al. 2009)].

In sum, our data establish MGMT as a potential new negative effector of GBM invasion beyond its well-known role in response to alkylating agents. Future preclinical studies are warranted to investigate the inverse relationship between MGMT and SPARC, and validate the prognostic value of MGMT and SPARC as new invasion biomarkers in prospective studies testing anti-invasive therapies to target invasive glioma cells with high levels of SPARC.

3.6. ACKNOWLEDGEMENTS

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Chapter 4: MGMT potentially regulates the migration/proliferation dichotomy in glioblastoma and increased invasiveness in response to angiogenic inhibitors

4.1. SUMMARY

The dichotomy between uncontrolled proliferation and excessive migration in glioblastoma multiforme (GBM) has posed unique implications for current standard therapy, making angiogenic inhibitors a promising therapeutic alternative. However, despite initial response to angiogenic inhibitors, GBM tumors tend to rebound with more aggressive growth.

We previously reported an inverse relationship between GBM invasion and the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT), and additionally showed that MGMT mediates increased sensitivity to treatment with the anti-angiogenic agent sunitinib. Therefore, in this study we investigated the role of MGMT in regulating the migration/proliferation dichotomy and the aggressive response to sunitinib and sorafenib treatment using overexpression and shRNA-mediated knockdown of MGMT and patient-derived cell lines. We found that MGMT overexpression in U87MG cells induced genetic alterations in proliferation-related processes, increased proliferation, and decreased expression of secreted protein acidic and rich in cysteine (SPARC), a proposed regulator of the migration/proliferation dichotomy. Conversely, knockdown of MGMT in T98G cells was associated with reduced proliferation and increased SPARC expression. Strikingly, we also noted that sunitinib and sorafenib treatment significantly decreased invasion of MGMT(+) cell lines, while invasion was increased in MGMT(-) cell lines. Furthermore, though treatment did not alter matrix metalloproteinase-2 activity or tissue inhibitor of metalloproteinase-1 expression in an MGMT-dependent manner, sunitinib did significantly reduce

proliferation of MGMT(+) cells compared to their isogenic MGMT(-) counterparts.

These results are the first to implicate MGMT as a potential regulator of the migration/proliferation dichotomy in GBM, and also highlight the effect of MGMT expression in suppressing an aggressive rebound response to anti-angiogenic treatment.

4.2. INTRODUCTION

Uncontrolled proliferation and extensive migration through the brain parenchyma (i.e. invasion) are biological hallmarks of glioblastoma multiforme (GBM), the most common and aggressive primary malignant brain tumor. Though these two processes work in concert to contribute to the rapid growth and infiltration of GBM, a growing body of evidence indicates that invasion and proliferation are mutually exclusive events, since highly motile GBM cells tend to have lower proliferation rates (Dalrymple, Parisi et al. 1994; Giese, Loo et al. 1996; Silbergeld and Chicoine 1997; Mariani, Beaudry et al. 2001). This phenomenon, termed the migration/proliferation dichotomy, is based on the hypothesis that since migratory and proliferative processes share common signaling pathways, a unique intracellular mechanism coordinates both behaviors (Giese, Bjerkvig et al. 2003). Though specific regulators of this dichotomy, such as epidermal growth factor receptor (EGFR) (Ghosh, Beas et al. 2010), SPARC (Schultz, Lemke et al. 2002), miR-145 (Godlewski, Nowicki et al. 2010), and ephrinB2 (Wang, Rath et al. 2012) have been elucidated, no regulator with clinically relevant application as a biomarker has been proposed.

Promoter methylation of the DNA repair protein O^6-methylguanine-DNA methyltransferase (*MGMT*) is a common predictive biomarker for increased sensitivity to temozolomide (TMZ) in GBM (Hegi, Diserens et al. 2005; Stupp, Hegi et al. 2009; Tabatabai, Stupp et al. 2010). Tumoral expression of MGMT mediates resistance to alkylating agent-induced cytotoxicity by removing alkyl lesions from the O^6 position of guanine (Esteller, Garcia-Foncillas et al. 2000). In

addition, promoter methylation of *MGMT* has been associated with a hypermutator phenotype encompassing mutations in several genes (Network 2008) that could influence tumor phenotype, including invasive and proliferative behavior. In this vein, evidence suggests that MGMT may play a role in determining the invasive and/or proliferative phenotype of several tumor types. For example, *in vitro* silencing of MGMT was associated with more aggressive motility and invasion of esophageal cancer cells (Su, Liu et al. 2011), while Konduri et al. established that inhibition of MGMT in pancreatic cancer cells resulted in reduced proliferation (Konduri, Ticku et al. 2009).

The migration/proliferation dichotomy in GBM poses major implications for therapy, as invasive cells with low proliferative potential are relatively resistant to conventional cytotoxic treatments directed against mitotically active cells (Roos and Kaina 2006). Thus, the utility of angiogenic inhibitors is a promising therapeutic alternative. Several growth factors and their cognate receptors are commonly overexpressed in GBM, leading to the formation of aberrant vascular networks in these tumors (Dunn, Heese et al. 2000). Sunitinib malate (Sutent) and sorafenib tosylate (Nexavar) are multi-targeted receptor tyrosine kinase inhibitors (TKIs) with anti-angiogenic activities that target numerous biologically relevant receptors in GBM including vascular endothelial growth factor receptor (VEGFR). Consequently, these agents can potentially impede GBM angiogenesis, migration, and proliferation, since VEGFRs and other receptor tyrosine kinases are implicated in numerous biological pathways (Ferrara 2004). Importantly, both sunitinib and sorafenib have demonstrated anti-

angiogenic and anti-tumor effects in preclinical GBM studies (Schueneman, Himmelfarb et al. 2003; de Bouard, Herlin et al. 2007; Siegelin, Raskett et al. 2010; Bareford, Park et al. 2011). However, clinical trials of angiogenic inhibitors for GBM suggest that despite initial responsiveness, the benefits of anti-angiogenic therapy are typically transitory, with no significant extension of overall survival (Neyns, Sadones et al. 2011; Reardon, Vredenburgh et al. 2011a; Reardon, Vredenburgh et al. 2011b). This rebound growth is mediated by several intrinsic or adaptive/evasive mechanisms, including the upregulation of invasive programs designed to evade hypoxia resulting from angiogenesis blockade (Bergers and Hanahan 2008; Norden, Young et al. 2008; Paez-Ribes, Allen et al. 2009; Keunen, Johansson et al. 2011).

Since GBM is notoriously heterogeneous and has recently been found to contribute to its own vascular supply (Ricci-Vitiani, Pallini et al. 2010; Wang, Chadalavada et al. 2010; Soda, Marumoto et al. 2011), GBM tumors may respond differently to anti-angiogenic treatment based on their phenotypic profiles. We previously showed that GBM cell lines expressing MGMT [ie. MGMT(+)] are molecularly and phenotypically different from MGMT(-) cell lines. Notably, we found that compared to MGMT(-) GBM cells, MGMT(+) cells exhibit decreased angiogenesis, increased sensitivity to sunitinib *in vitro* (Chahal, Xu et al. 2010), and reduced invasion (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012). Therefore, we investigated whether the relationship between MGMT expression and GBM invasion translates to an inverse relationship with proliferation as predicted by the migration/proliferation dichotomy, and whether

MGMT influences the rebound response to anti-angiogenic treatment with sunitinib or sorafenib. Using isogenic overexpression and knockdown of MGMT in established GBM cell lines, as well as primary patient-derived cell lines with differing MGMT status, we found that MGMT(+) cells are more proliferative than MGMT(-) as predicted by the migration/proliferation dichotomy, and have a differential response profile to angiogenic inhibitors.

4.3. MATERIALS AND METHODS

4.3.1. Cell culture

U87-MG empty vector (U87/EV) and its MGMT-transfected derivative U87/MGMT (Aghi, Rabkin et al. 2006), as well as T98G empty vector (T98/EV) and its stable MGMT knockdown derivative T98shC1.1 (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012) were maintained at 37ºC, 95% humidified air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S) (Invitrogen) (standard media).

Patient-derived primary GBM cell lines (P-GM1, P-GM2, P-GM5, P-GM7) were kindly provided by Dr. Kenneth Petruk (University of Alberta) and Dr. Joan Turner (Cross Cancer Institute), and were maintained in DMEM/F12 supplemented with 10% FCS, 1% P/S, and 1% glutamate (Invitrogen).

4.3.2. In vitro drug treatment

Sunitinib malate (SU, Pfizer) and sorafenib (SF, Bayer) were dissolved in dimethylsulfoxide (DMSO). Cells were serum-starved in DMEM containing 0.5% FCS overnight, then exposed to SU (1 μM) for 2 h or SF (1 μM) for 48 h in standard media prior to assays. Following the 2 h treatment with SU, cells were incubated overnight in standard media prior to assays. DMSO was used as a control for SU and SF experiments. For analysis of SPARC methylation, cells were treated with 5-aza-2'-deoxycytidine (5-aza, Sigma) (5 μM) in standard media and replenished daily for 4 days.

4.3.3. Proliferation assay

Cells were seeded (following treatment when indicated) in a 96-well plate (500 cells/well) for 48 h, and proliferation was assessed using the XTT Cell Proliferation Kit (Roche) following manufacturer's instructions.

4.3.4. Invasion assay

In vitro cell invasion was measured using BD BioCoat Matrigel invasion chambers (BD Biosciences; 8 μm pore sizes) following manufacturer's instructions. Following treatment, cells (2.5×10^4) were seeded in top chambers with DMEM containing 0.5% FCS and allowed to invade towards a chemoattractant (DMEM + 10% FCS) for 24 h. Drugs were added to the top and bottom chambers. The membranes were then fixed with 3.7% formaldehyde and stained with 1% crystal violet. Invasive cells were visualized by bright field microscopy using a Zeiss Axioskop2 Plus microscope attached to an Axiocam color camera with a Zeiss FLUAR 5x/0.25 lens and counted using MetaMorph 7.7 software.

4.3.5. Western blotting

Following treatment, cells were washed twice with phosphate-buffered saline (PBS) and lysed with RIPA buffer (20 nM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM NaPP, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin) (Schueneman, Himmelfarb et al. 2003). 30 μg of protein (BCA protein assay kit, Pierce) were separated by 12% SDS-PAGE under

reducing conditions and transferred onto polyvinylidine difluoride membranes. Membranes were probed for MGMT (BD Biosciences), secreted protein acidic and rich in cysteine (SPARC, Cell Signaling), or β-actin (Sigma-Aldrich). Densitometric analysis (Adobe Photoshop CS3) shows relative band intensities normalized to levels of b-actin.

4.3.6. Gelatin zymography

The identification of matrix metalloproteinase-2 (MMP-2) activity was performed using gelatin zymography by electrophoresis of serum-free conditioned media collected from cells 24 h following drug treatment. The loading amounts of protein were normalized by the number of cells in each plate, and samples in 2x Novex® Tris-Glycine SDS Sample Buffer (Invitrogen) were loaded without reduction in 10% SDS polyacrylamide gels supplemented with 0.1% gelatin. Following electrophoresis at constant voltage of 125V for 90 min, gels were washed in renaturing buffer (2.5% Triton X-100 in H_2O) to remove SDS and renature the MMPs, and incubated overnight at 37ºC in Novex® Developing Buffer (Invitrogen) to induce gelatin lysis by MMPs. Gels were stained with Coomassie brilliant blue R-250 (Sigma), and destained with a solution of 10% ethanol and 7.5% acetic acid.

4.3.7. Enzyme-linked immunosorbent assay (ELISA)

Cells growing at 70% confluency were treated as described above. The resultant conditioned medium was collected and passed through a 0.22 μm filter

to remove cell debris. TIMP-1 analysis (R&D Systems) was performed according to manufacturer's instruction. TIMP-1 concentrations were calculated from standard curves generated using recombinant human TIMP-1. Protein concentration was normalized by the number of cells in each plate.

4.4. RESULTS

4.4.1. Genes involved in proliferation are differentially regulated in MGMT(+) GBM cells

We have previously shown that MGMT expression is inversely correlated with invasion in glioblastoma (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012). Considering the inherent dichotomy between invasion and proliferation in GBM cells (Giese, Loo et al. 1996; Berens and Giese 1999), we sought to determine whether MGMT expression influences the proliferative capacity of GBM cells. First, we utilized a previously conducted cDNA microarray of the MGMT(-) GBM cell line U87MG (transfected with an empty vector construct: U87/EV) and its clone stably transfected with MGMT (U87/MGMT) to determine the effect of MGMT overexpression on the expression of other genes (Chahal, Xu et al. 2010). Data analysis using the Gene Ontology (GO) Consortium (Ashburner, Ball et al. 2000) revealed that genes involved in the regulation of cell proliferation are significantly differentially expressed between the two cell lines (Table 4.1). Notably, expression of murine double minute 4 (*MDM4*/*HDMX*), which enhances cellular proliferation by inhibiting p53-mediated transcriptional activation of *p21* (Jin, Cook et al. 2010), was increased in U87/MGMT cells compared to their MGMT(-) counterpart. In addition, erbB2 (*ERBB2*, HER2/neu), a related protein and preferential dimerizing partner of EGFR that induces proliferation (Kristt and Yarden 1996; Andersson, Guo et al. 2004), was also increased in U87/MGMT cells. Thus, gene expression

analysis revealed a dramatic switch in the proliferative profile of U87MG cells based on MGMT expression.

Table 4.1. Differential regulation of GO biological processes involved in proliferation.

Biological Processes involved in proliferation

Term	Count	% of gene list	P value
GO:0042127~regulation of cell proliferation	67	2.99%	0.011294034
GO:0007050~cell cycle arrest	14	0.62%	0.074115791
GO:0008283~cell proliferation	93	4.14%	0.079240095
GO:0051726~regulation of cell cycle	64	2.85%	0.082519791

4.4.2. MGMT(+) GBM cells are more proliferative and express less SPARC than MGMT(-) cells

To determine how alterations in proliferation-related genes manifest at a functional level, we assessed *in vitro* proliferation of U87/EV, U87/MGMT, the MGMT(+) GBM cell line T98G (stably transfected with an empty vector construct: T98/EV), and its stable shRNA-mediated MGMT knockdown clone T98shC1.1 (Figure 4.1A). As described previously, the invasive potential of the MGMT(+) cell lines U87/MGMT and T98/EV were lower than their MGMT(-) counterparts U87/EV ($P < 0.001$) and T98shC1.1 ($P = 0.046$), respectively (Figure 4.1B). Conversely, using the XTT proliferation assay we established that cellular proliferation of U87/MGMT cells was increased 2.80-fold compared to U87/EV cells ($P < 0.001$), while the proliferation rate of T98/EV cells was 1.83-fold higher than that of T98shC1.1 ($P = 0.020$) (Figure 4.1C). Therefore, MGMT appears to dichotomously regulate invasion and proliferation of GBM cells.

To further assess the potential role of MGMT in the migration/proliferation dichotomy, we examined the expression of SPARC in our cell lines. Increased expression of SPARC, a glycoprotein that inhibits cell adhesion to the extracellular matrix (ECM) (Sage 1997), is correlated with increased invasion but decreased cell proliferation in GBM, and is therefore a putative regulator of the migration/proliferation dichotomy (Schultz, Lemke et al. 2002; Seno, Harada et al. 2009). Of note, we previously reported a correlation between immunohistochemical SPARC expression and *MGMT* promoter methylation in a series of GBM biopsies, suggesting an inverse relationship

between MGMT and SPARC (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012). Western blotting analysis revealed a 92% reduction of SPARC expression in the MGMT-overexpressing U87/MGMT cell line compared to U87/EV, and an 89% increase of SPARC expression in MGMT-knockdown T98shC1.1 cells compared to T98/EV cells (Figure 4.1D).

SPARC promoter methylation is one of the potential mechanisms of SPARC protein regulation (Suzuki, Hao et al. 2005; Socha, Said et al. 2009). Therefore, we next determined whether methylation was responsible for the loss of SPARC expression in our MGMT(+) cell lines by treating cells with the demethylating agent 5-aza-2'-deoxycytidine (5-aza, 5 μM for 4 days). Treatment with 5-aza was able to restore SPARC expression in the U87/MGMT cell line, which experienced an 83% increase in expression, and further increased SPARC expression by 30% in the highly expressing U87/EV cell line. However, 5-aza treatment did not significantly alter expression of SPARC in T98/EV or T98shC1.1 cells (Figure 4.1E).

Taken together, these results show that overexpression of MGMT is correlated with increased proliferation, decreased invasion, and decreased expression of SPARC in accordance with the migration/proliferation dichotomy. Furthermore, MGMT regulation of SPARC may be dependent on promoter methylation.

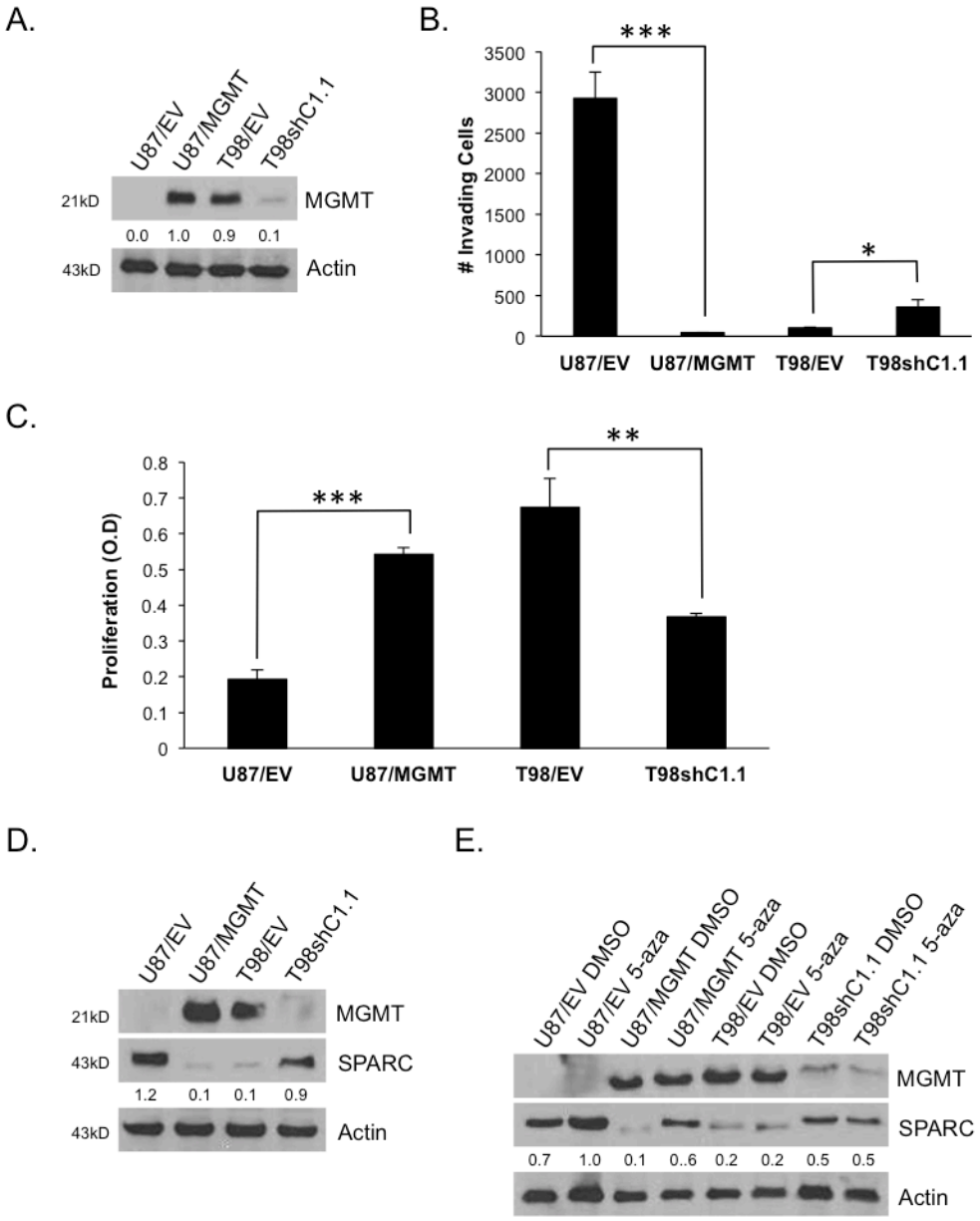

Figure 4.1. Relationship between MGMT expression, invasion, proliferation, and SPARC expression in GBM cell lines. (A) Western blotting analysis shows MGMT protein levels normalized to actin in U87MG cells stably transfected with empty vector (U87/EV) or MGMT (U87/MGMT), and T98G stably transfected with empty vector (T98/EV) control or MGMT-shRNA (T98shC1.1). **(B)** Histogram shows the number of invading cells assessed by Matrigel invasion assay. **(C)** Histogram shows the rate of proliferation in arbitrary units of cell lines. (Means ± SEM fold increase; n = 3; **P* < 0.05, ***P* < 0.01, ****P* < 0.0001) **(D)** Western blotting analysis of basal expression of SPARC and **(E)** expression of SPARC following DMSO or 5-aza treatment normalized to actin. Note that the overexpression of MGMT is associated with dichotomous regulation of invasion and proliferation, and decreased expression of SPARC.

4.4.3. Treatment with sunitinib and sorafenib significantly increases invasion in MGMT(-) GBM cells, while invasion of MGMT(+) cells is decreased

Though angiogenic inhibitors are a potential therapeutic alternative for GBM, treatment is often limited by aggressive rebound growth commonly mediated by increased cell invasiveness. We therefore investigated whether our previously reported relationship between MGMT expression and GBM invasion influences the invasive response to anti-angiogenic therapy by assessing invasion of the isogenic MGMT overexpressing and knockdown cell lines following treatment with sunitinib [1 μM for 2 h, as described previously (Chahal, Xu et al. 2010)] and sorafenib [1 μM for 48 h, as described (Siegelin, Raskett et al. 2010)]. Strikingly, U87/EV cells exhibited a significant 1.50-fold increase in invasion following sunitinib treatment (P = 0.021), while invasion of U87/MGMT cells was decreased by 2.53-fold (P = 0.046). Additionally, sunitinib treatment significantly decreased invasion of T98/EV (1.47-fold, P = 0.006), while invasion of T98shC1 cells was increased (1.69-fold, P = 0.003) (Figure 4.2A). The cell lines responded analogously to sorafenib treatment, with invasion of U87/EV cells increasing 2.35-fold (P = 0.028), invasion of U87/MGMT decreasing 1.95-fold (P = 0.047), and invasion of T98/EV cells decreasing by 2.53-fold (P = 0.009). However, sorafenib did not appear to alter the invasiveness of T98shC1.1 cells (P = 0.877) (Figure 4.2B).

To validate these findings and determine their potential clinical applicability, we examined the invasive response to sunitinib and sorafenib in 4 primary patient-derived cell lines: the MGMT(-) P-GM1 and P-GM2 cell lines

that exhibit high basal level invasion, and the MGMT(+) P-GM5 and P-GM7 cell lines that are less invasive, as described previously (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012) (Figure 4.3A). Following sunitinib treatment MGMT(-) cells (P-GM1 and P-GM2) exhibited significant increased invasion ($P = 0.041$ and $P = 0.048$, respectively), while invasion of MGMT(+) cells (P-GM5 and P-GM7) was significantly decreased ($P = 0.019$ and $P = 0.012$) (Figure 4.3B). Similarly, both MGMT(-) cell lines responded to sorafenib treatment with significantly increased invasion (P-GM1 displayed a 1.48-fold increase, $P = 0.032$; P-GM2 displayed a 1.37-fold increase, $P = 0.016$), while invasion of one of the MGMT(+) cell lines P-GM5 was significantly decreased by 1.54-fold ($P = 0.032$) (Figure 4.3C).

Therefore, MGMT status not only influences the invasive and proliferative phenotype of GBM cells, but also appears to affect the invasive response to anti-angiogenic treatment with the multi-targeted TKIs sunitinib and sorafenib.

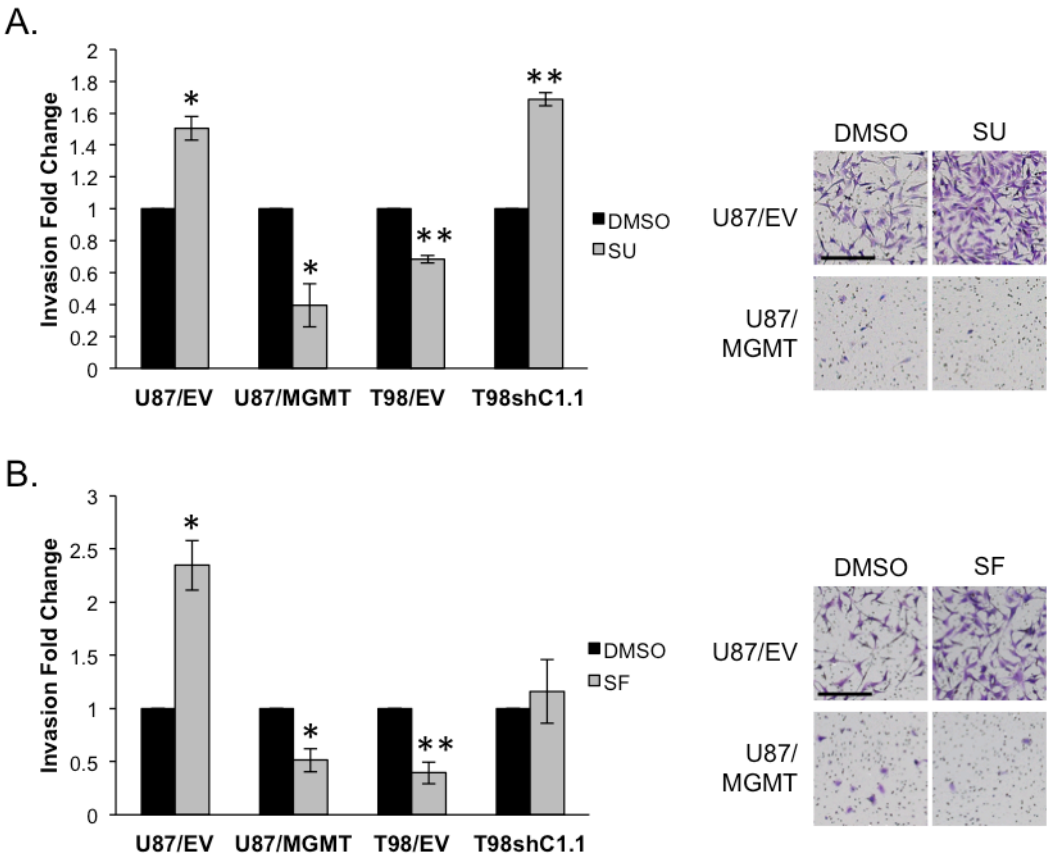

Figure 4.2. Invasion increases following sunitinib and sorafenib treatment in MGMT(-), but not in MGMT(+) isogenic GBM cell lines. Histograms show the differential effect of **(A)** sunitinib (SU, 1 μM, 2 h) and **(B)** sorafenib (SF, 1 μM, 48 h) compared to DMSO control on invasion of isogenic cell lines as determined by Matrigel invasion assay. (Means ± SEM fold increase; n = 3; **P* < 0.05, ***P* < 0.01). Representative photomicrographs highlight increased invasion of U87/EV cells, while invasion of U87/MGMT cells decreased following treatment. Scale bar, 300 μm.

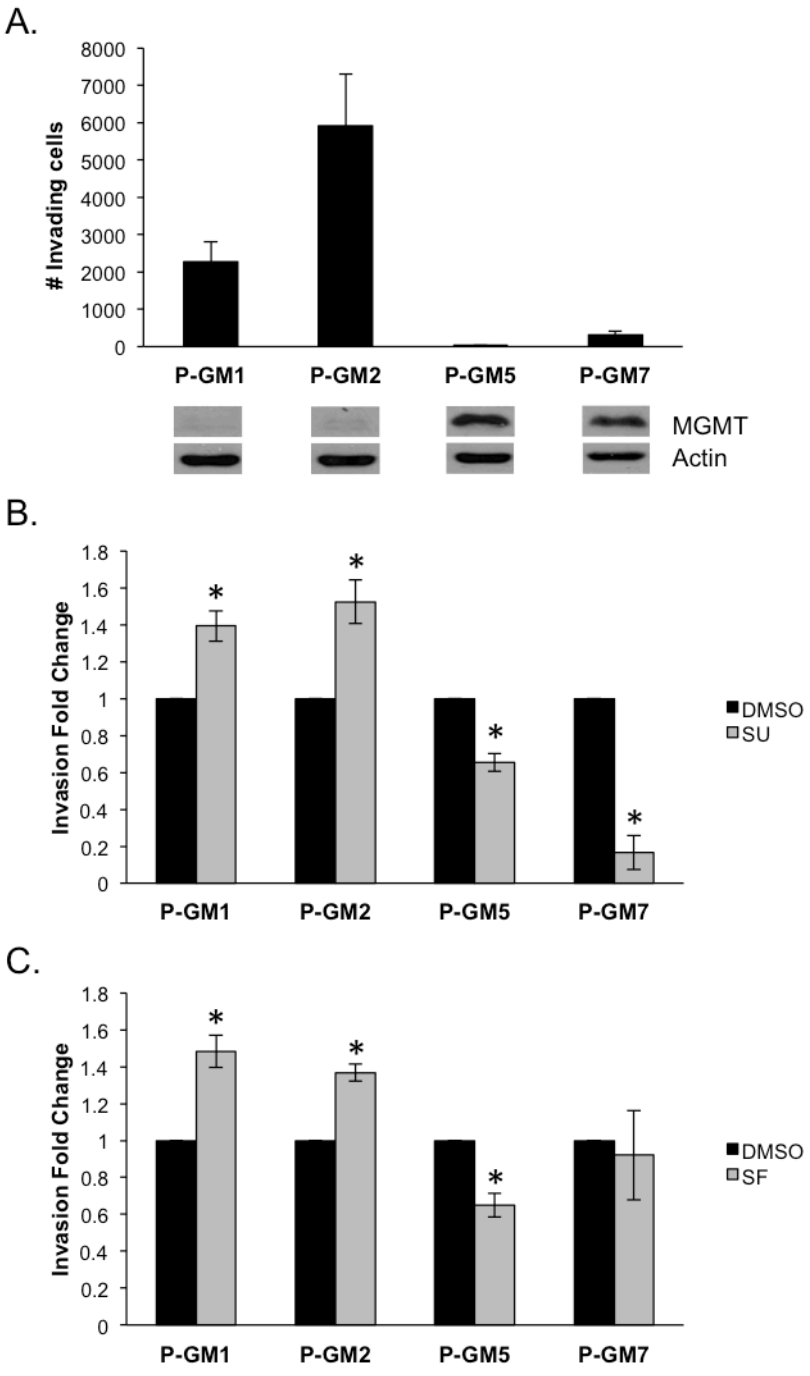

Figure 4.3. Invasion increases following sunitinib and sorafenib treatment in MGMT(-), but not in MGMT(+) primary patient-derived GBM cell lines. (A) Histogram shows the number of invading cells of 4 primary cell lines assessed by Matrigel invasion assay. Immunoblots show MGMT protein levels and actin loading control. Histograms show the differential effect of **(B)** sunitinib (SU, 1 μM, 2 h) and **(C)** sorafenib (SF, 1 μM, 48 h) compared to DMSO control on invasion of primary cell lines as determined by Matrigel invasion assay. (Means ± SEM fold increase; n = 3; $*P < 0.05$)

4.4.4. Expression of MMP-2 and TIMP-1 following sunitinib and sorafenib treatment

As we observed a stark difference in invasive response to multi-targeted TKIs based on MGMT status, we next aimed to identify potential mediators of this differential invasive response. Matrix metalloproteinase-2 (MMP-2) plays a fundamental role in degrading ECM proteins, which is required for increased GBM invasion (Nakada, Okada et al. 2003) as well as normalization of abnormal tumor vasculature during anti-angiogenic treatment (Winkler, Kozin et al. 2004). To evaluate whether enzymatic activity of MMP-2 was differentially regulated based on MGMT status following sunitinib and sorafenib treatment, we performed gelatin zymography using supernatant from the four isogenic cell lines with and without treatment. Importantly, MMP-2 activity was highest in U87/EV cells, which are most invasive. Additionally, MMP-2 activity was noticeably increased following both treatments in the U87/EV cell line, correlating with the increased invasiveness observed. In contrast to the invasive behavior of U87/MGMT cells, MMP-2 activity was increased following sunitinib treatment. However, MMP-2 activity did not reflect the alterations in invasive capacity of the other cell lines, as activity was not significantly different between treatment and control conditions (Figure 4.4A).

We additionally examined the secretion of tissue inhibitor of metalloproteinases-1 (TIMP-1) following treatment. Though TIMP-1 is a naturally occurring inhibitor of most MMPs, it is likely a multi-potent protein with other important functions. Of note, GBM patients with high TIMP-1 protein

expression have shorter overall survival compared to patients with low expression (Aaberg-Jessen, Christensen et al. 2009), and increased TIMP-1 expression has been correlated with increased GBM invasion following anti-angiogenic treatment with bevacizumab *in vitro* (Lucio-Eterovic, Piao et al. 2009). Contrary to the observations of Lucio-Eterovic et al., we observed that following treatment with multi-targeted TKIs, secretion of TIMP-1, as determined by ELISA assay, was decreased in U87/EV cells ($P = 0.004$ for sunitinib treatment, $P = 0.048$ for sorafenib treatment). Though TIMP-1 was not significantly altered upon sunitinib treatment in the other cell lines, sorafenib treatment induced a decrease in TIMP-1 secretion in T98/EV and T98shC1.1 cell lines ($P = 0.008$ and $P = 0.04$, respectively). Interestingly, control treated conditions revealed a trend of increased TIMP-1 secretion in MGMT(-) cell lines compared to their corresponding MGMT(+) counterparts (Figure 4.4B and C).

These results suggest that MMP-2 and TIMP-1 may play a role in the differential invasiveness of these cell lines, but may only mediate response to angiogenic inhibition in U87/EV cells.

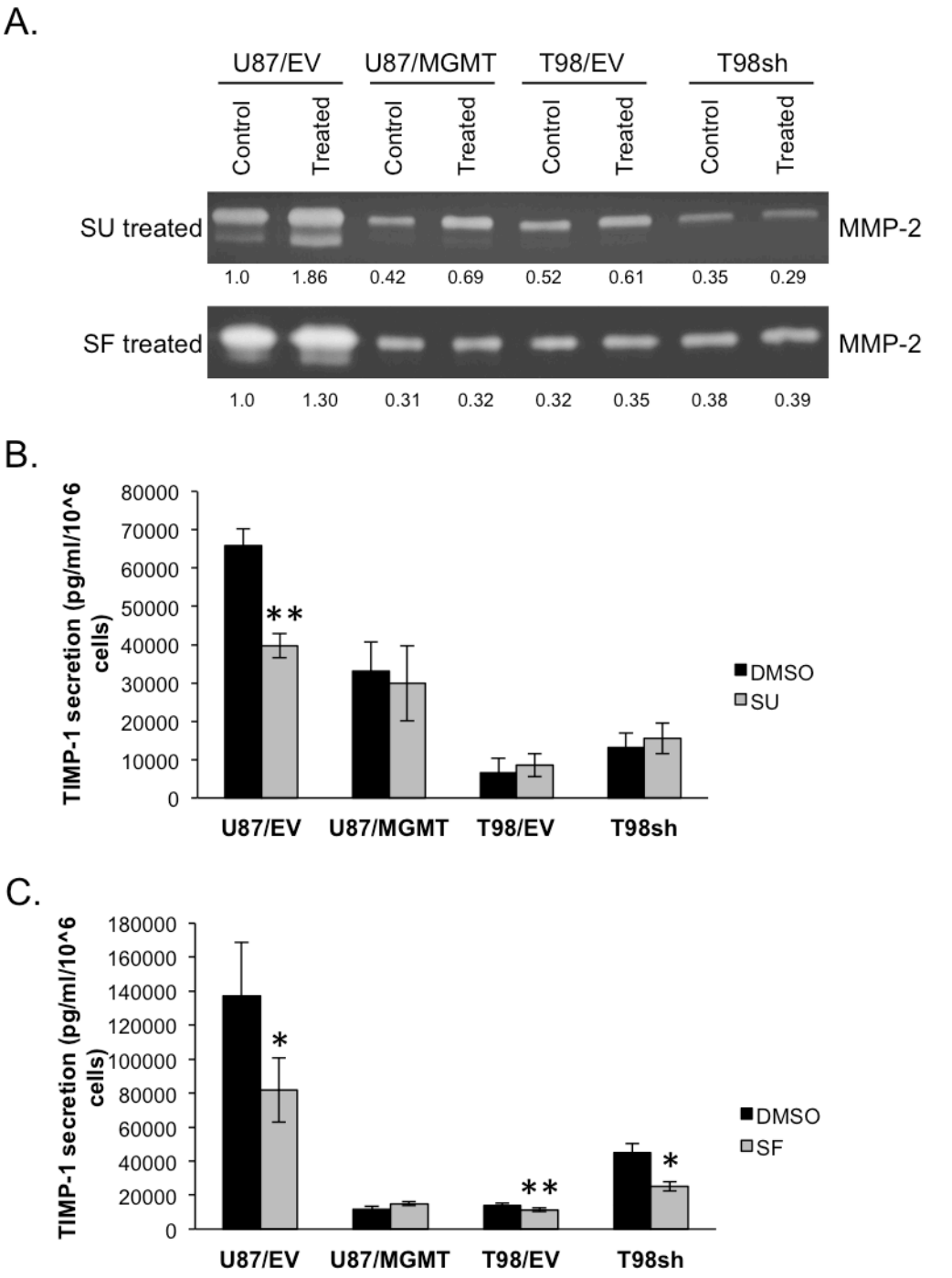

Figure 4.4. MMP-2 activity and TIMP-1 secretion following sunitinib and sorafenib treatment. (A) Gelatin zymography for MMP-2 using conditioned media from DMSO control and sunitinib (SU, 1 μM, 2 h) or sorafenib (SF, 1 μM, 48 h) treated cells. The loading amounts were normalized by the number of cells in each plate. Secretion of TIMP-1 following **(B)** sunitinib and **(C)** sorafenib compared to DMSO control. (Means ± SEM fold increase; n = 3; **P* < 0.05, ***P* < 0.01)

4.4.5. Proliferation of GBM cells following sunitinib and sorafenib treatment

To assess whether the observed relationship between invasion, proliferation, and MGMT expression influences response to angiogenic inhibitors, we next investigated proliferation of MGMT(+) and MGMT(-) cell lines after sunitinib and sorafenib treatment. Sunitinib treatment did not dramatically alter proliferation of the MGMT(-) cell lines U87/EV or T98shC1.1 compared to control conditions. However, in comparison to their MGMT(-) counterparts, the MGMT(+) cell lines U87/MGMT and T98/EV experienced significantly reduced proliferation ($P < 0.001$ and $P = 0.037$, respectively) when exposed to sunitinib (Figure 4.5A). Although a similar trend in proliferative response was apparent after treatment with sorafenib, there was no statistically significant difference between treatment in MGMT(-) versus MGMT(+) cell lines (Figure 4.5B). Consequently, sunitinib appears to evoke more of a differential proliferative response in GBM cell lines based on MGMT status compared to sorafenib.

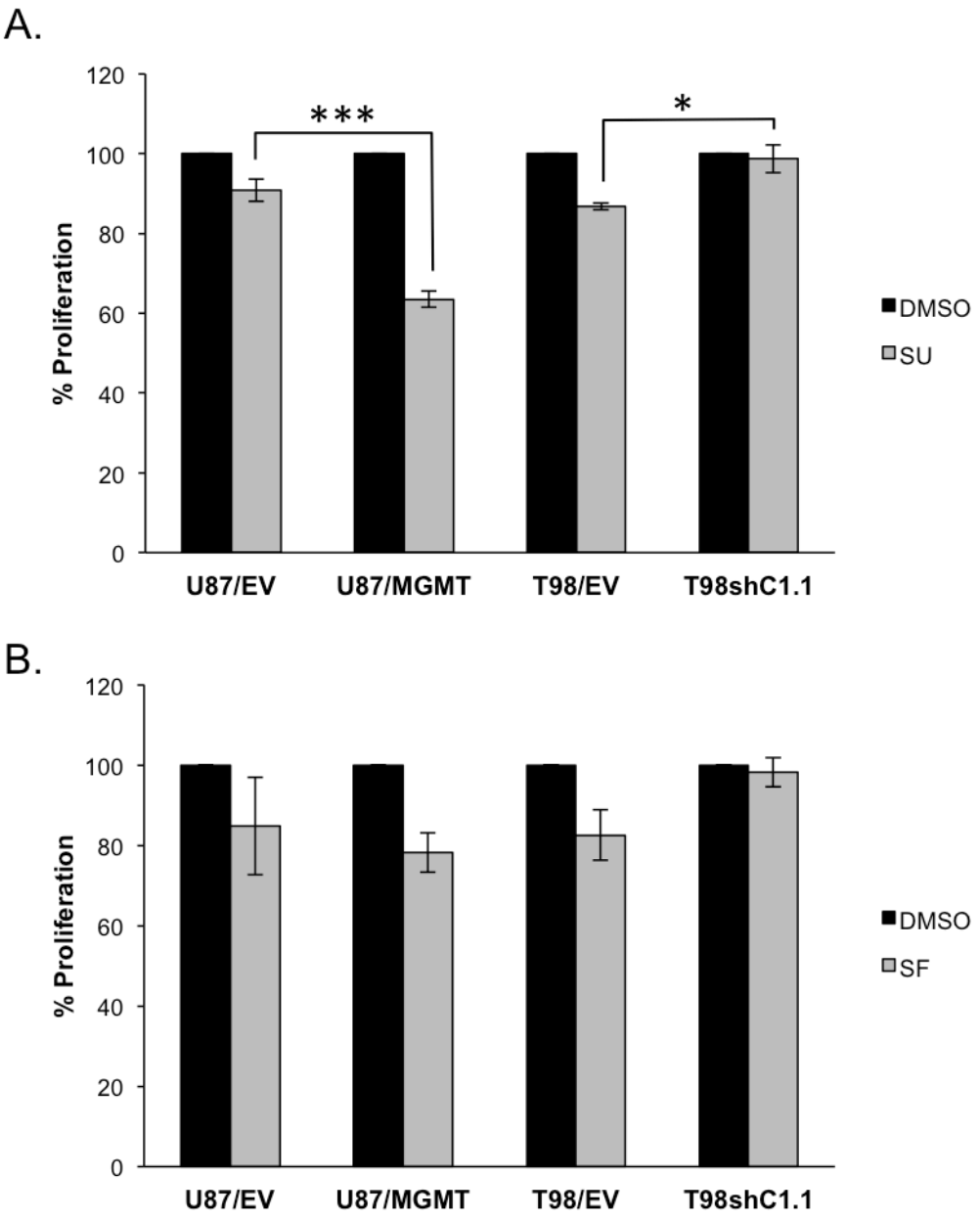

Figure 4.5. Proliferation is significantly lower in isogenic MGMT(+) cells compared to MGMT(-) cells in response to sunitinib, but not sorafenib. Effect of **(A)** sunitinib (SU, 1 μM, 2 h) or **(B)** sorafenib (SF, 1 μM, 48 h) on proliferation of isogenic cell lines relative to DMSO control. (Means ± SEM fold increase; n = 3; *$P < 0.05$, ***$P < 0.001$)

4.5. DISCUSSION

In this study, we provide the first evidence of novel roles for MGMT in the regulation of GBM proliferation and in mediation of aggressive rebound response to angiogenic inhibitors. Using isogenic and primary patient-derived GBM cell lines differing in MGMT protein expression, we show that while MGMT expression increases cell proliferation, it also confers greater sensitivity to treatment with sunitinib or sorafenib, as only cells deficient in MGMT experience a rebound invasive response to angiogenic blockade.

Our previous observations indicated that MGMT(+) GBM cells are less angiogenic, less tumorigenic (Chahal, Xu et al. 2010), and less invasive (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012) compared to MGMT(-) cells. However, the prognosis for GBM patients is extremely poor regardless of MGMT status, suggesting that both MGMT(+) and MGMT(-) tumors are highly aggressive. Moreover, from a therapeutic perspective MGMT(-) tumors have a better prognostic outcome compared to MGMT(+) tumors because of their increased sensitivity to treatment with TMZ (Hegi, Diserens et al. 2005) and, as was recently observed, radiotherapy alone (Rivera, Pelloski et al. 2010). Therefore, to account for the clinical aggressiveness of MGMT(+) tumors, we proposed that MGMT(+) cells may compensate for decreased invasion by upregulating alternative growth mechanisms. In accordance with the well-described migration/proliferation dichotomy (Tektonidis, Hatzikirou et al. 2011; Hatzikirou, Basanta et al. 2012), our data revealed that while invasion was decreased, proliferation of MGMT(+) cell lines was higher than their MGMT(-)

counterparts. Specifically, gene expression profiling analysis revealed alterations in cell growth and survival pathways and increased expression of the proliferation inducers *ERBB2* and *MDM4* in MGMT(+) cells. Functional *in vitro* analysis of overexpression and knockdown models further demonstrated that MGMT promoted cell proliferation. The potential role of MDM4, an inhibitor of p53-dependent regulation of cell cycle progression, in mediating the increased proliferative potential of MGMT(+) cells is of particular interest. Considering the varied methods of cross-talk thought to occur between MGMT and wild-type or mutant p53 (Grombacher, Eichhorn et al. 1998; Esteller, Risques et al. 2001; Bocangel, Sengupta et al. 2009), further investigation is required to determine if MGMT influences GBM proliferation through a p53-dependent mechanism.

Although the migration/proliferation dichotomy has been well documented in GBM, its underlying cellular and molecular mechanisms are largely unknown. The observation that SPARC inversely correlates with MGMT expression in our cell lines lends greater support to our hypothesis that MGMT influences the migration/proliferation dichotomy. High expression of SPARC is associated with suppressed proliferation but enhanced migration and invasion of GBM through mediation of integrins including β_1 integrin (Weaver, Workman et al. 2008), growth factor receptor-regulated kinases such as integrin-linked kinase and focal adhesion kinase (Shi, Bao et al. 2007), and their downstream effectors (Thomas, Alam et al. 2010). The reduction of SPARC upon forced expression of MGMT, and concurrent increase in SPARC expression upon shRNA-mediated knockdown of endogenous MGMT, suggests that MGMT may act as a master regulator of the

migration/proliferation dichotomy by modulating SPARC. Our initial data using the demethylating agent 5-aza indicates that MGMT may suppress SPARC by epigenetic silencing via promoter methylation in some cell lines. However, because 5-aza treatment only rescued SPARC expression in one of two MGMT(+) cell lines (U87/MGMT, but not in T98/EV) and also amplified expression in the MGMT(-) cell line U87/EV exhibiting high basal levels of SPARC protein, further investigation into the mechanism of SPARC regulation by MGMT is required. Of note, VEGF has also been reported to bind to SPARC, resulting in an increase in SPARC mRNA and protein levels (Kato, Lewalle et al. 2001) and reduced VEGFR-mediated proliferation signaling (Kupprion, Motamed et al. 1998) in endothelial cells. Therefore, in T98/EV cells, which did not respond to 5-aza treatment and which we previously reported have low VEGF expression (Chahal, Xu et al. 2010), SPARC may be regulated through a VEGF-dependent mechanism. Thus, MGMT may also potentially increase proliferation of GBM cells by reducing both VEGF and SPARC expression.

While these findings establish the relevance of MGMT in the migration/proliferation dichotomy, the potential clinical significance of our study is further exhibited in our investigations using angiogenic inhibitors. As illustrated in overexpression, knockdown models, and primary GBM cell lines, our data suggesting that sunitinib or sorafenib treatment exacerbated the invasive phenotype mostly in MGMT(-) cells are unprecedented and intriguing. This is of considerable interest in the context of preclinical and clinical studies reporting limited therapeutic efficacy of anti-angiogenic therapies resulting from tumor

progression or increased invasion (Paez-Ribes, Allen et al. 2009; Neyns, Sadones et al. 2011; Reardon, Vredenburgh et al. 2011a). We were unable to conclusively determine if MMP-2 activity or TIMP-1 activity contributed to the differential invasiveness of MGMT(+) versus MGMT(-) cells following therapy, possibly due to low basal invasiveness of U87/MGMT, T98/EV, and T98shC1.1 cell lines. However, one might speculate that these inhibitors target a negative effector of invasion expressed in MGMT(-) cells whereby effective inhibition of this target may lead to increased invasion. MGMT expression may also prevent escape from angiogenic inhibition by regulating expression of VEGF, as we previously reported (Chahal, Xu et al. 2010). Anti-angiogenic treatment has been shown to escalate cell invasiveness by generating an intratumoral hypoxic environment and upregulation of hypoxia inducible factor-1α (HIF-1α) (Keunen, Johansson et al. 2011; Conley, Gheordunescu et al. 2012), that can subsequently induce upregulation of VEGF (Shweiki, Itin et al. 1992). Since VEGF expression is also important in resistance to angiogenic inhibitors, low basal VEGF expression in MGMT(+) cells may preclude a rebound aggressive response, while high VEGF expression in MGMT(-) cells equips them for heightened aggressiveness following treatment.

Indeed, multi-targeted TKIs often exhibit moderate to high affinity for additional kinases beyond their primary intended targets (Karaman, Herrgard et al. 2008), which could modulate the cellular and anti-tumor activity of these agents. By targeting a variety of receptor tyrosine kinases possessing mitogenic activity, in our *in vitro* studies it seems that TKIs may display greater anti-tumor

activity in highly proliferating MGMT(+) cell lines compared to the relatively quiescent MGMT(-) cell lines. Accordingly, the enhanced activity of sunitinib compared to sorafenib in reducing proliferation of MGMT(+) cell lines may be explained by the recent discovery that sunitinib has a broader kinase inhibiting activity than sorafenib (Kumar, Crouthamel et al. 2009). Thus, along with our previous study showing increased *in vitro* sensitivity of U87/MGMT cells to sunitinib compared to U87/EV cells (Chahal, Xu et al. 2010), our current findings highlight the rationale for potential use of multi-targeted TKIs in MGMT(+) GBM patients, with caution against their utility in MGMT(-) GBM patients due to deleterious effects (i.e. increased invasiveness) observed in MGMT(-) cell lines. Results from clinical trials testing combined treatments using the anti-invasive agent cilengitide in GBM patients with a methylated promoter of *MGMT* (2008) and more recently in patients without methylation (2010) may provide additional insights with respect to the relationship between MGMT and invasion/proliferation of GBM following anti-angiogenic therapy.

Inhibition of angiogenesis using anti-angiogenic therapeutics may not always elicit an evasive tumoral response (Ebos and Kerbel 2011), and the impetus for enhanced disease progression following angiogenic inhibition may depend on the molecular and phenotypic profile of individual tumors. Our study identifies MGMT expression as a potential mechanism underlying increased sensitivity to treatment with multi-targeted TKIs, and as an important modulator of GBM phenotype and aggressive modality. Thus, MGMT expression is a promising criterion for the development of targeted therapies for GBM. Combined

with our previous findings in archived patient tumors that *MGMT* methylation (i.e. protein silencing) correlates with increased expression of SPARC (Chahal et al. *Mol Cancer Ther*. Submitted revision, MCT-11-0977R, 2012), our current study emphasizes the potential use of MGMT as a biomarker for patient selection in clinical trials using angiogenic inhibitors. Further mechanistic and preclinical studies are warranted to elucidate the extent of MGMT influence on GBM beyond mediation of resistance to alkylating agents.

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Chapter 5: Conclusions and Perspectives

5.1. PRIMARY FINDINGS

Despite significant advances in neuroimaging, surgery, radiation therapy, and chemotherapy, survival figures for patients with GBM have not dramatically improved in the last 60 years. Thus, transforming GBM into a curable disease requires new paradigms in GBM biology and improved understanding of the mechanisms underlying GBM aggression, treatment resistance, and recurrence.

To this effect, promoter methylation of the DNA repair gene *MGMT* has emerged as the most powerful predictive marker of sensitivity to standard therapy with TMZ (von Deimling, Korshunov et al. 2011), and as a strong prognostic factor dictating outcome in patients with newly diagnosed GBM undergoing chemotherapeutic treatment (Criniere, Kaloshi et al. 2007; Colman and Aldape 2008; Olson, Brastianos et al. 2011). Unfortunately, this knowledge has not yet translated into improved therapeutic options for the 60-75% of GBM patients with treatment-resistant tumors expressing MGMT (Chamberlain 2010). All patients regardless of MGMT status currently receive standard treatment with TMZ. Therefore, more than half of patients with GBM derive no benefit from chemotherapy, resulting in unnecessary depletion of resources, financial burden, and risk of TMZ-associated side effects.

Emerging evidence suggests that although decreased expression of MGMT contributes to TMZ sensitivity, it may also contribute to tumor progression by enabling the acquisition of several mutations. Accordingly, we believe that our work elucidating a new role for MGMT in GBM aggressiveness

and response to anti-angiogenic treatment could potentially result in novel and significant clinical applications.

5.1.1. Analysis of experimental models

In our efforts to delineate novel functional roles for MGMT in GBM we primarily used an *in vitro* model system consisting of the commonly utilized MGMT(-) U87MG glioblastoma cell line (Clark, Homer et al. 2010) stably transfected with empty vector (U87/EV) or MGMT [U87/MGMT, provided by Dr. Manish Aghi (Aghi, Rabkin et al. 2006)]. We supplemented this overexpression model with stable shRNA-mediated knockdown of MGMT in the MGMT(+) T98G glioblastoma cell line, generating T98shRNA clones with reduced MGMT expression. When compared to their parental cell lines, U87/MGMT and T98shRNA cell lines enabled us to accurately analyze the effect of MGMT manipulation on GBM. Because stable RNA interference (RNAi) can potentially instigate off-target effects in the cell (Martin and Caplen 2007), we additionally used O6BG, a substrate analog of MGMT that induces its degradation (Dolan, Moschel et al. 1990), to assess short-term effects of MGMT depletion. Functional validation of MGMT overexpression and knockdown was accomplished by establishing their decreased and increased sensitivity to TMZ, respectively, relative to parental cell lines. To validate some of our findings in a more clinically relevant model, we utilized a panel of low-passage patient-derived primary GBM cell lines, since primary cell lines replicate the *in vivo* behavior of GBM more accurately than established cell lines (Ashley, Riffkin et al. 2008).

Together, these models permitted the identification of correlative and causal associations between MGMT expression and alterations in GBM phenotype and behavior.

5.1.2. The role of MGMT in GBM pathology

To the best of our knowledge, our investigations are the first to describe MGMT as an important regulator of the GBM phenotype (summarized in Table 5.1). Notably, gene expression profiling revealed that induced MGMT expression resulted in differential expression of over 3000 genes involved in numerous biological processes. These altered processes included pathways regulating angiogenesis, invasion, and proliferation, which constitute the hallmarks of GBM aggression. Expanding on this discovery, molecular analysis of VEGF and its receptors as well as *in vitro* tube formation assay indicated that MGMT expression inversely correlates with angiogenic potential. A recent report by Della Puppa et al. corroborated our finding by determining that the highly angiogenic peripheral region of GBM tumors, which expresses high levels of VEGF, also exhibits reduced expression of MGMT relative to inner regions displaying low VEGF (Della Puppa, Persano et al. 2011).

MGMT expression additionally correlated with reduced GBM invasiveness as determined by i) reduced integrin and FAK expression, ii) reduced motility, iii) altered morphology, and iv) reduced *in vitro* invasion using a panel of established and primary cell lines. Conversely, reducing MGMT resulted in increased invasion. We further showed an inverse relationship between MGMT

and the pro-invasive protein SPARC in a series of primary GBM biopsy tumors, as there was a significant correlation between *MGMT* promoter methylation and immunohistochemical protein expression of SPARC. Our data are therefore consistent with previous studies in other cancer models suggesting an inverse correlation between MGMT and invasiveness (Park, Han et al. 2001; Takeshita, Inoshita et al. 2009; Su, Liu et al. 2011).

Considering the widely accepted principle that an inherent inverse association between invasion and proliferation exists in GBM (Giese, Loo et al. 1996; Hatzikirou, Basanta et al. 2012), our observation that MGMT overexpression increases proliferation while MGMT knockdown decreases proliferation is particularly intriguing. The opposing regulation of invasion and proliferation by MGMT, along with reduced expression of SPARC in MGMT(+) cell lines compared to their MGMT(-) counterparts, suggests that MGMT expression may regulate the migration/proliferation dichotomy and shift the mode of aggression of GBM cells. A potential corollary of this finding is that heterogeneous MGMT expression throughout a GBM tumor may influence intratumoral pathological heterogeneity. Studies of GBM heterogeneity indicate that cells in the enhancing region of the tumor mass are more proliferative while peripheral cells are highly invasive and spread throughout the brain parenchyma (Glas, Rath et al. 2010; Molina, Hayashi et al. 2010). Recent studies have also suggested that MGMT expression progressively decreases from the inner to the peripheral concentric layer of the tumor (Pistollato, Abbadi et al. 2010; Della

Puppa, Persano et al. 2011). Our study is the first to suggest a potential association between these observed features of GBM.

Our investigations also revealed significantly reduced tumorigenic potential of MGMT(+) cell lines compared to MGMT(-) cell lines, which may be rationalized by the reduced angiogenic and invasive profile of these cells. While the importance of VEGF expression in tumor formation is well established (Kim, Li et al. 1993; Oka, Soeda et al. 2007), an *in vivo* chronological analysis recently highlighted the additional necessity of early invasion events in tumor initiation. Sampetrean et al. (2011) reported that upon orthotopic implantation of BTICs in mice, the first events associated with gliomagenesis were tumor cell migration along fiber tracts and perivascular infiltration, followed by the emergence of nuclear atypia, and finally, mass tumor formation characterized by heightened proliferation and necrosis (Sampetrean, Saga et al. 2011). Similarly, a previous study also suggested that tumor cells must first migrate to suitable vascular regions to enable subsequent proliferation through environmental cues (Farin, Suzuki et al. 2006). Thus, we can surmise that despite their heightened proliferation, our MGMT(+) cell lines were unable to initiate tumors because of their reduced infiltrative and angiogenic capacity.

Notably, our data implicating MGMT as a regulator of the migration/proliferation dichotomy may also account for the inevitable resistance to TMZ that occurs in patients with GBM despite MGMT status. As a cytotoxic chemotherapeutic agent, TMZ is most effective in cells that are actively proliferating. However, if rapidly proliferating cells express high levels of

MGMT, the efficacy of TMZ will be limited. Conversely, invasive cells with decreased MGMT expression are expected to be more susceptible to TMZ because of decreased repair capacity, but because these cells exhibit reduced proliferation the therapeutic efficacy of TMZ would not be optimal in MGMT(-) cells. Therefore, our study indicating a regulatory role for MGMT in GBM phenotype emphasizes the requirement of pursuing alternative therapeutic options for this highly aggressive disease.

Table 5.1. Summary of relative phenotypic differences between MGMT(-) and MGMT(+) GBM cells.

	MGMT(-)	MGMT(+)
Angiogenesis		
VEGF-A and VEGFR-2 Expression	High	Low
VEGFR-1 and sVEGFR-1 Expression	Low	High
Induction of Endothelial Tube Formation	High	Low
Invasion		
in vitro Invasion	High	Low
Integrin and FAK expression	High	Low
in vitro Motility	High (Mesenchymal)	Low
Morphology	Mesenchymal	Round
SPARC Expression in Tissue Samples **	High	Low
Proliferation		
SPARC expression	High	Low
in vitro Proliferation	Low	High
Tumorigenicity		
Subcutaneous Murine Model	Tumorigenic	Non-Tumorigenic

**SPARC expression in patient biopsy tumors correlated with *MGMT* methylation, but not MGMT protein expression

5.1.3. The role of MGMT in response to angiogenic inhibitors

Because MGMT plays a crucial role in resistance to TMZ, and because GBM invariably progresses following TMZ treatment regardless of MGMT status, we investigated the potential role of MGMT in dictating tumor response to anti-angiogenic therapy with multi-targeted TKIs. Previous analyses of the anti-angiogenic agent cilengitide, an integrin inhibitor, in combination with RT and TMZ revealed a benefit only to GBM patients with *MGMT* methylated tumors, while MGMT levels *in vitro* did not induce a differential response to cilengitide alone (Maurer, Tritschler et al. 2009). In contrast, our results indicated preferential activity of the TKI sunitinib in MGMT(+) cell lines compared to their MGMT(-) counterparts as determined by analysis of i) *in vitro* proliferation, ii) clonogenic viability, and iii) ERK and AKT signaling pathway inhibition. Additional studies are warranted to validate the potential use of MGMT as a biomarker of response to angiogenic inhibitors such as sunitinib.

The elucidation of a novel role for MGMT in response to angiogenic inhibitors has compelling implications for future development of molecularly targeted therapeutics for GBM. Although signal transduction inhibitors have not yet been proven to be highly effective treatments for GBM, the enthusiasm for their use in GBM derives from the remarkable success of selected kinase inhibitors for cancers such as chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). The disparity in success rate for such treatments in CML and GIST versus GBM may stem from the fact that the primary molecular therapeutic targets are present in an overwhelming majority of

CML and GIST patients, while genetic alterations not readily detected by routine pathological examinations are present in varying combinations in GBM (Huang, Sarkaria et al. 2009). MGMT is one of the few molecular markers commonly assessed in GBM (Camara-Quintana, Nitta et al. 2012). Therefore, the prospect that MGMT status can influence response to molecularly targeted therapy with agents like sunitinib is particularly promising.

Additionally, our finding that sunitinib exhibits more potent antitumor effects in MGMT(+) cell lines compared to MGMT(-) cell lines can help define subpopulations of GBM patients for which anti-angiogenic therapy is more effective. Most clinical trials thus far have been conducted in unselected patient populations, and a positive response in a subset of patients with molecular similarities can be diluted when combined with non-responders in the whole population (Huse, Phillips et al. 2011). This dilution was evident in early trials for the EGFR inhibitor gefitinib for non-small-cell-lung cancer. Fortunately, following initial negative results conducted in unselected patients, highly positive results were seen in selected patients with EGFR-mutant tumors (Reck 2009). Accordingly, a lack of patient stratification in clinical trials may prematurely cease further investigation of potentially efficient anti-angiogenic agents before the identification of therapeutically relevant markers. In this vein FDA approval for bevacizumab as a treatment for metastatic breast cancer was recently revoked due to the inability to verify the improvement in progression free survival seen in the original trial, and because the benefit/risk ratio was deemed unacceptable (Tanne 2011). Bevacizumab was also granted accelerated approval for recurrent

GBM in 2009, but because numerous recent trials in unselected patient populations indicate only transitory response followed by progressive and highly infiltrative disease (Norden, Young et al. 2008; Narayana, Kelly et al. 2009; de Groot, Fuller et al. 2010), its utility in GBM may be destined for the same outcome as seen in breast cancer. Consequently, our observation that sunitinib and sorafenib treatment only exacerbate invasion in MGMT(-) cells while reducing invasion in MGMT(+) cells is highly relevant. This finding highlights the deleterious effect of anti-angiogenic agents in MGMT(-) tumors, and may therefore lend credence to our claim that angiogenic inhibitors could potentially be a suitable therapeutic alternative only for patients with MGMT(+) GBM tumors, who currently derive little benefit from standard therapy with TMZ.

5.2. FUTURE DIRECTIONS

Our study demonstrates novel roles for MGMT in GBM phenotype and response to multi-targeted TKIs. These observations lay important groundwork for further development in deciphering the complexities of GBM, in the continuing pursuit of improved therapeutic interventions for GBM, and in expanding our knowledge of MGMT, which was previously considered to only possess the function of repairing alkyl lesions.

5.2.1. In vitro investigations

Though serum-cultured established and primary patient-derived cell lines are extensively used for *in vitro* analyses of GBM, it is well recognized that GBM-derived neurospheres more effectively preserve the molecular and phenotypic characteristics of the tumor from which they originate (Lee, Kotliarova et al. 2006; De Witt Hamer, Van Tilborg et al. 2008). This is mostly due to the ability of neurosphere culture to enrich for a BTIC subpopulation (Singh, Hawkins et al. 2004; Yuan, Curtin et al. 2004). Though there is contention regarding the expression of MGMT in BTICs (Beier, Rohrl et al. 2008; Blough, Westgate et al. 2010; Pistollato, Abbadi et al. 2010; He, Shan et al. 2011), focusing on these highly relevant cells would help further elucidate the role of MGMT in this complex tumor type. Thus, in an ongoing effort to validate our findings and clarify the differential effect of MGMT on GBM angiogenesis, invasion, and proliferation, a reasonable next step would be to conduct functional *in* vitro assays in neurospheres derived from fresh GBM biopsy samples. BTICs

have also proven to be especially important in resistance to anti-angiogenic therapeutics as they express increased levels of pro-angiogenic factors (Bao, Wu et al. 2006; Folkins, Shaked et al. 2009) and can differentiate into endothelial progenitors to renew tumoral vascular supply (Wang, Chadalavada et al. 2010). Consequently, neurospheres also represent a unique tool to assess the effect of MGMT expression on response to angiogenic inhibitors. Along with primary cell lines, these neurospheres can be used to generate a larger panel of cell line pairs with differential MGMT expression by transfecting with MGMT or shRNA directed towards MGMT (Ying, Sang et al. 2011).

Our cDNA microarray using U87/EV and U87/MGMT cells identified over 3 000 genes differentially expressed based on MGMT status. To streamline the identification of pathways altered as a consequence of MGMT expression, we can conduct additional gene expression profiling of T98/EV and T98shC1.1 and other cell line pairs. Subsequent MGMT-based clustering analysis using all cell lines would enable the definition of a molecular signature associated with MGMT status. Specifically, we can define the role of MGMT in the migration/proliferation dichotomy by establishing whether genes involved in biological processes associated with migration, invasion, and proliferation are differentially expressed in our panel of cell lines.

Indeed, our finding that MGMT may modulate the migration/proliferation dichotomy is highly intriguing and warrants further evaluation. Our *in vitro* analysis of the dichotomy can be advanced by utilizing a 2D wound healing assay (Liang, Park et al. 2007) and examining migration and proliferation at the wound

edge by live cell imaging of our MGMT-modulated cell line pairs. The extent of migration will be determined by measuring the area of the wounded region lacking cells at each time point, while proliferation will be measured by determining the ratio of cells that undergo successful division to the total number of cells at the wound edges. Moreover, we can expand our preliminary data implicating SPARC as a potential mediator of MGMT influence on the migration/proliferation dichotomy by investigating its regulatory mechanisms. Treatment with 5-aza suggested that SPARC might be regulated by methylation in our U87/EV and U87/MGMT cell lines. Our lab is currently investigating the differential methylation of genes based on MGMT status in our isogenic cell lines and in MGMT(+) primary cell lines treated with O6BG. From this analysis conducted by Genome Quebec we can determine if SPARC and other genes are regulated by methylation in an MGMT-dependent manner. Furthermore, since SPARC may also be regulated by VEGF (Kupprion, Motamed et al. 1998; Kato, Lewalle et al. 2001), our data showing that MGMT also influences VEGF expression suggests that further investigation into the relationship between MGMT, VEGF, and SPARC may be of value.

In addition, our cDNA microarray identified the differential expression of *ERBB2*, a protein related to EGFR, based on MGMT expression. Because ErbB2, EGFR, and its truncated mutant EGFRvIII are implicated in regulation of GBM invasion and proliferation (Huang, Nagane et al. 1997; Tsatas, Kanagasundaram et al. 2002; Ghosh, Beas et al. 2010), analysis of these receptors in our cell lines is a logical avenue of study. Our finding that *MDM4*, an inhibitor of p53-dependent

regulation of cell proliferation, is also upregulated in MGMT(+) cells suggests that the p53 pathway may influence the regulation of the migration/proliferation dichotomy by MGMT. Considering the proposed relationships between MGMT and p53 (Srivenugopal, Shou et al. 2001; Bello, Alonso et al. 2004; Bocangel, Sengupta et al. 2009), manipulating p53 expression in MGMT(+) and MGMT(-) cell lines may lend further insight into the mechanism of invasion and proliferation regulation by MGMT.

Due to the potential value of utilizing angiogenic inhibitors in GBM, our data indicating that MGMT(+) cell lines respond more favorably to treatment with TKIs also merit additional assessment. Since our initial investigation of TIMP-1 and MMP-2 expression did not fully elucidate why MGMT(-) cells exhibit increased invasion following treatment, we must further investigate the mechanism of invasive response to anti-angiogenic treatment. Quantitative real-time reverse transcription PCR arrays specific for cell motility, wound healing, or ECM proteins can identify potential mediators of this response by screening for changes in mRNA levels of invasion-related genes. Furthermore, since bevacizumab is an FDA approved treatment for recurrent GBM, investigation of response based on MGMT status is highly relevant. Although we conducted preliminary *in vitro* studies using bevacizumab in our cell lines (data not shown), our results were unreliable due to the high concentration (Lucio-Eterovic, Piao et al. 2009) and consequently high ratio of drug volume to media volume required. Additional investigation into response of MGMT(+) and (-) GBM cells to

bevacizumab can help us determine if the differential response we see using anti-angiogenic TKIs translates to angiogenic inhibition by an alternative method.

5.2.2. In vivo investigations

Although our *in vitro* investigations and proposed strategies provide a vital foundation for subsequent studies, they have notable limitations. In particular, they are unable to reproduce the cerebral environment that likely represents a unique determinant for the aggressive phenotype of GBM and its response to angiogenic inhibition (Hoelzinger, Demuth et al. 2007). To translate our findings to a more appropriate physiological setting, we will determine whether differential MGMT expression is correlated with a different GBM phenotypic profile or response to angiogenic inhibitors *in vivo* using a murine orthotopic model.

Importantly, though U87MG cells notoriously generate tumors with profuse neovascularization (Candolfi, Curtin et al. 2007), necrotic foci are rare and tumors show a non-diffusely infiltrative growth pattern that is uncharacteristic of GBM (Jacobs, Valdes et al. 2011). Because of the inability of U87MG cells to develop tumors that recapitulate most of the key salient features of GBM, and because our data indicate that U87/MGMT cells have low tumorigenicity (even in an intracranial model), alternative cell lines must be utilized. By stereotactically implanting primary cell lines or neurosphere-derived cells with MGMT overexpressed or knocked down into the cerebrums of immunocompromised mice, we can investigate the effect of MGMT expression on GBM phenotype or

response to treatment in a model that accurately reflects the histopathological heterogeneity of GBM in a relevant environment.

In our MGMT(+) and (-) cell lines we will also enforce expression of the firefly luciferase protein, which produces bioluminescent light from the substrate D-luciferin. Intracranial injection of these cells into mice along with intraperitoneal injection of D-luciferin will provide a means to visualize tumor growth. To assess the effect of MGMT expression on tumor growth patterns and the efficacy of angiogenic inhibitors, we will monitor several parameters including tumor growth by live cell imaging, survival of mice, and circulating levels of angiogenesis and invasion markers in blood (such as VEGF, SPARC, and YKL-40). Post-mortem tumors will be further analyzed for histopathologic evaluation using hematoxalin and eosin (H/E) and IHC staining (Candolfi, Curtin et al. 2007) to analyze expression of MGMT, angiogenesis markers VEGF and CD34, invasion markers SPARC and YKL-40, and the proliferation index by staining for Ki-67 (Braun, Papadopoulos et al. 1988). The anti-tumor effect of angiogenesis inhibitors will be evaluated by assessing the number of mice showing tumor regression, tumor growth delay, and Kaplan-Meyer analysis to determine the effect on morbidity. This study could provide the rationale to investigate the use of MGMT as a biomarker in clinical studies testing the efficacy of angiogenic inhibitors in GBM patients.

5.2.3. Identifying a mechanism for novel MGMT actions

Our clonogenic assay testing TMZ-sensitivity of different clones derived from T98G MGMT-knockdown indicated that though derivative cell lines displayed altered invasive profiles based on MGMT levels, they responded similarly to TMZ treatment. This finding suggests that MGMT regulation of GBM phenotype may not be related to its alkyltransferase activity. To date the only known function of MGMT is the transfer of alkyl adducts at the O^6 position of guanine to an internal cysteine residue (cys-145) in its active site (Grafstrom, Pegg et al. 1984; Gerson 2004). To conclusively ascertain whether MGMT mediates GBM phenotype via its alkyltransferase activity, we can generate cell lines with mutated MGMT active sites by site-directed mutagenesis of the cys-145 residue, and subsequently determine if MGMT-mutant cells have a similar phenotype to MGMT(+) or (-) cells. Conformational changes in the MGMT protein resulting from alkylation at the active site cys-145 initiate its subsequent ubiquitylation and proteasomal degradation. Moreover, MGMT may be interacting with DNA or proteins in an unknown manner. Therefore it is vital that an active site mutant does not significantly alter the structure of the MGMT protein (Hazra, Roy et al. 1997; Xu-Welliver and Pegg 2002). Transfection of MGMT(-) cells with a construct harboring *MGMT* mutations and assessing their effects on the angiogenic, invasive, and proliferative profile will provide new insights into how MGMT may affect phenotypic and biological aspects, and will reveal whether those functions could be achieved independently from the alkyltransferase activity of MGMT or not.

Furthermore, the fact that MGMT acts alone while repairing alkylating damage does not preclude the possibility that MGMT may work in concert with other proteins to execute its novel functional activities in GBM. Specifically, functional and physical interactions of MGMT with binding partners may account for the alterations of the transcriptome, functional pathways, and biological effects we observe in MGMT(+) cells. In this vein, modified MGMT having undergone a conformational change following repair of an alkyl lesion, has been found to bind with estrogen receptor, which consequently inhibits cell proliferation (Teo, Oh et al. 2001). By using a functional proteomic approach we can assess if and how MGMT interacts with other proteins to regulate angiogenesis, invasion, and proliferation. Although a proteomic analysis of MGMT has been previously conducted (Niture, Doneanu et al. 2005), the mechanism of MGMT functional diversity has never been studied in a GBM background. The identification of novel MGMT binding proteins can be accomplished by tandem mass spectrometry (Wang and Li 2008) or tandem affinity purification (Burckstummer, Bennett et al. 2006), which are able to detect proteins present in low concentrations or that interact with only a fraction of cellular MGMT. Following confirmation of selected proteins involved in our processes of interest by co-immunoprecipitation with MGMT, this approach may provide mechanistic insight into functional activities of MGMT in GBM.

5.2.4. Clinical validation

To draw the most meaningful conclusions from our findings for the clinical setting, we must examine the correlation between MGMT and GBM phenotype in paraffin archived tissues derived from surgical biopsies. Expanding upon our findings that SPARC expression inversely correlates with MGMT in a panel of paraffin-embedded GBM tumor specimens, we can verify the role of MGMT in the migration/proliferation dichotomy by concurrent staining with Ki-67 for cell proliferation assessment. The determination of MGMT influence on GBM angiogenesis is also currently being assessed in our paraffin-embedded tumor samples of GBM patients (collaboration with Dr. Jay Easaw and Dr. Tony Magliocco of the Tom Baker Cancer Centre). Using the HistoRx's automated quantitative analysis (AQUA) technology, which combines fluorescence-based imaging with high-throughput automated microscopy (Le, Harris et al. 2009), assessment of VEGFR-1 and -2 and VEGF-A was performed for 78 of 268 tumor samples. In this preliminary study, of the 18 patients with unmethylated *MGMT* promoter regions, 17 were scored as "VEGFR-1 high", which correlated with our *in vitro* findings in the MGMT(+) cell lines. A high-risk of relapse in *MGMT* methylated tumors was also associated with high VEGF-A and low VEGFR-1. Further evaluation of additional markers relating to angiogenesis, invasion, and proliferation may provide better insight into the relationship between MGMT status and GBM phenotype in patients.

Within paraffin blocks, tumor cells can be isolated from histologically normal brain by macrodissection or laser-capture microdissection (LCM) (Espina,

Wulfkuhle et al. 2006) for *in vitro* validation of biomarkers. LCM uses a specialized microscope-guided laser to isolate specific cells of interest from a heterogeneous population. The highly precise extraction process does not alter the morphology or chemistry of the samples or surrounding cells (Espina, Wulfkuhle et al. 2006), thereby making it a useful method of collecting cells for DNA and RNA analyses. mRNA can be extracted from the dissected tumor cells to corroborate IHC results by QRT-PCR.

Additionally, our preclinical determination of preferential sensitivity in MGMT(+) cells to angiogenic inhibition with multi-targeted TKIs (particularly sunitinib) warrants validation in prospective clinical studies. Accordingly, in collaboration between the Cross Cancer Institute, the Tom Baker Cancer Centre and McGill University, our lab will launch a phase II clinical trial of concurrent sunitinib, TMZ, and RT followed by adjuvant sunitinib for newly diagnosed GBM patients specifically with unmethylated *MGMT* promoters (study sponsored by Pfizer Inc., ethical approval McGill University 1132REB). This study will investigate tumor response and progression free survival of the MGMT(+) subgroup of patients compared to a historical cohort of GBM patients treated with standard of care. Furthermore, correlative translational studies aim to identify patients most likely to respond to sunitinib-based therapy by analyzing systemic, circulating, and imaging biomarkers to evaluate the efficacy and safety of sunitinib during treatment. This study will provide the proof-of-principle to design optimal clinical trials testing anti-angiogenic therapy in GBM based on clinically relevant biomarkers.

5.3. CONCLUSIONS

To date, *MGMT* promoter methylation is the only biomarker used to predict the efficacy of TMZ in GBM patients. Although patients with tumors lacking MGMT expression are more sensitive to treatment with TMZ, the prognosis for patients with both MGMT(+) and (-) tumors is still extremely poor. Our investigations are the first to describe MGMT as a potential mediator of GBM angiogenesis, invasion, and proliferation, thereby highlighting novel roles for this previously well-characterized protein in cancer. Moreover, the possibility that MGMT status could predict a subset of patients that respond more favorably to multi-targeted TKIs is an encouraging starting point for further investigation of the utility of angiogenic inhibitors in GBM. Therefore, our study provides new insight into this complex and deadly tumor, and constitutes an important step towards developing more effective therapeutic options for patients suffering with GBM.

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Appendix A: Addition of sunitinib to standard treatment does not significantly improve tumor growth delay of U87/EV xenografts

A.1. METHODS- Tumor growth and treatment in mice

For growth delay assays following treatment, U87/EV cells (5 x 10^6) suspended in 150 μL serum-starved DMEM media were injected subcutaneously in the right flank of Balb/c *nu/nu* mice. Following tumor formation, mice were randomized into 4 groups (vehicle control, sunitinib+RT, TMZ+RT, sunitinib+ TMZ+RT) of 10 mice in each group. Irradiated mice were immobilized in acrylic chambers, and the entire body was shielded with lead except for the tumor-bearing hind limb. Radiation (3 doses of 5 Gy on days 1-3) was administered within 30 min of sunitinib (40 mg/kg) and/or TMZ (5 mg/kg) intraperitoneal (i.p) injection and followed by daily i.p injections of sunitinib and/or TMZ 5 days a week for 4 weeks. All experiments were approved by the Cross Cancer Institute Animal Care Committee and performed under institutional guidelines in accordance with approved regulatory standards.

A.2. RESULTS

A recent study showed that the anti-angiogenic activity of sunitinib was associated with increased survival of mice bearing intracerebral U87MG tumors (de Bouard, Herlin et al. 2007). To our knowledge, the effect of sunitinib in combination with standard treatment (RT + TMZ) has not yet been studied. We investigated whether sunitinib compared to standard treatment affects tumor growth delay in athymic Balb/C *nu/nu* mice subcutaneously injected with U87/EV cells. Due to the decreased tumorigenicity of U87/MGMT cells, tumor growth delay could not be assessed in an MGMT(+) GBM xenograft model. Mice were treated with combinations of sunitinib, RT, and TMZ as described in materials and methods. Tumor growth was drastically impaired with the standard treatment compared to mice injected with vehicle control (DMSO). The addition of sunitinib to RT or RT + TMZ also significantly reduced tumor growth in comparison with DMSO ($P = 0.03$ and $P = 0.009$, respectively) without any additional toxicity, but did not significantly improve tumor growth delay compared to the standard treatment (RT + TMZ, $P = 0.4$ and $P = 0.2$, respectively) by the end of the treatment schedule (Figure A.1).

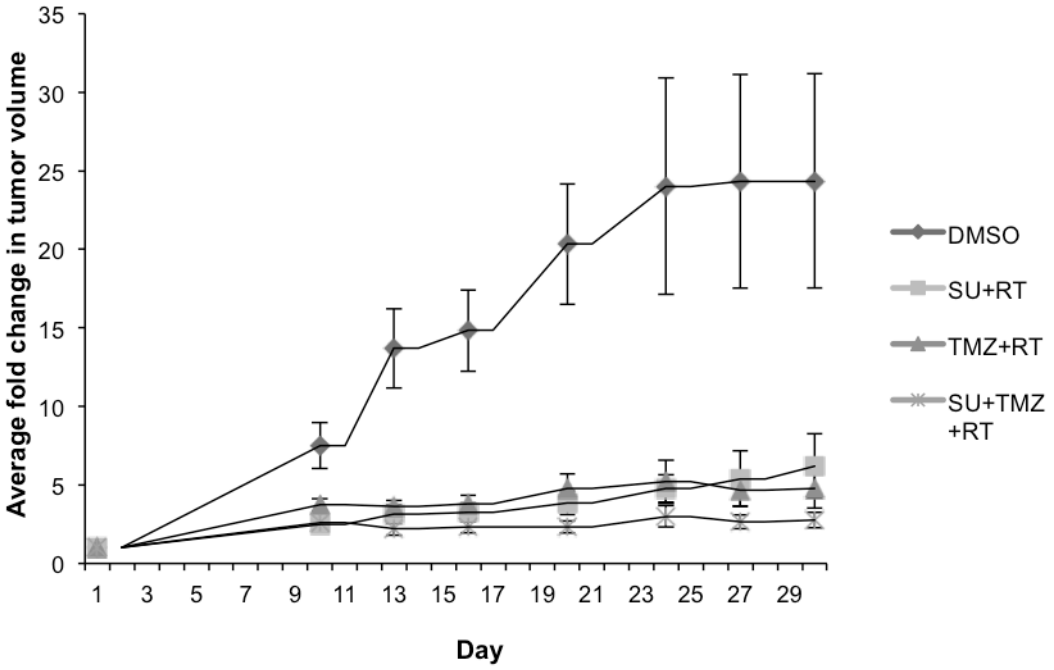

Figure A.1. Tumor growth curve of U87/EV xenografts following treatment. U87 cells (5 x 10^6) were injected subcutaneously in the flanks of Balb/c nu/nu mice. Once tumors were established they were treated with 6 Gy on 3 consecutive days then injected intraperitoneally with 40 mg/kg sunitinib and/or 5 mg/kg TMZ for 5 days/week over 4 weeks, Tumor growth was analyzed using digital caliper measurements. Shown are the mean changes in tumor volume in 10 mice/treatment group.

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