THE INFLUENCES OF MATRIX METALLOPROTEINASE-1 EXPRESSION ON GLIOBLASTOMA PATHOLOGY

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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## List of Common Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>BCNU</td>
<td>bis-chloronitrosourea</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Related Kinases</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N&lt;sub&gt;ω&lt;/sub&gt;-Nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Murine Double Minute</td>
</tr>
<tr>
<td>MGMT</td>
<td>O&lt;sup&gt;6&lt;/sup&gt;-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane Type MMP</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric Oxide Synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinases</td>
</tr>
<tr>
<td>PAR-1</td>
<td>Protease Activated Receptor-1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble Guanylate Cyclase</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium Nitroprusside</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline Tween-20</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Abstract

THE INFLUENCES OF MATRIX METALLOPROTEINASE-1 EXPRESSION ON GLIOBLASTOMA PATHOLOGY

By Nicholas Alexander Pullen, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010.

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Glioblastoma multiforme (GBM) is an aggressive central nervous system (CNS) cancer characterized by enhanced tumor cell motility, pernicious invasion into the normal brain, extensive tumor-induced angiogenesis, and adaptive resistance to current therapeutic paradigms. One of the difficulties associated with GBM is the ability of the tumor cells to infiltrate normal CNS tissue. Neurosurgeons can remove the primary tumor mass, but
peripheral cells that are inaccessible will ultimately result in a secondary lesion that can lead to death.

The matrix metalloproteinases (MMP) are well known for their abilities to facilitate processes of cellular motility and invasion through their clearance of extracellular matrix (ECM). A specific member of this family, MMP-1, is not observed in normal brain, yet its expression is a common characteristic of GBM. The various causes of MMP-1 expression, and its consequences in GBM cells are unknown.

As such, functional studies were conducted related to the induction of MMP-1 expression via another molecule intrinsic to GBM, nitric oxide (NO). The exposure of GBM cell lines to nanomolar concentrations of NO produced significant inductions of MMP-1 expression and GBM cell motility. The specific removal of MMP-1 with siRNA elicited an abrogation of NO-stimulated motility, suggesting a pathological contribution by this enzyme.

Furthermore, recent accumulating evidence suggests that MMP-1 contributes to tumor cell survival and related angiogenesis in other cancer settings. To investigate these capabilities in GBM, cell lines were stably engineered to have either MMP-1 over-expression or knock-down. Both tumor formation and size were significantly reduced with MMP-1 knock-down and significantly increased with over-expression. In a
model of GBM cell-induced angiogenesis, the presence of MMP-1 contributed to an angiogenic phenotype. Further angiogenesis studies revealed a significant recruitment of host endothelium to the tumor interstitium in vivo. Proteomic studies suggest that one mechanism by which MMP-1 could influence angiogenesis is through the easement of the anti-angiogenic tissue inhibitor of metalloproteinases-4 (TIMP-4), since the removal of MMP-1 elicits a significant increase in TIMP-4 detection.

Altogether, these functional data present MMP-1 as a promising target for future therapeutic investigation, because it is unique to the GBM environment and contributes to key overlapping GBM pathologies.
Chapter 1

Introduction
I.1 Central Nervous System Neoplasia

Over eighty years have elapsed since the collaborative work of Percival Bailey and Harvey Cushing, *A Classification of Tumors of the Glioma Group on a Histogenic Basis with a Correlated Study of Prognosis*, which pioneered the consensus needed to better understand and study cancers of the central nervous system (CNS, Ferguson and Lesniak, 2005). Since then the World Health Organization (WHO) has published four ‘blue book’ guides specifically detailing CNS neoplasia (Louis et al., 2007). The first of these reports (Zülch, 1979) established a basic, widely accepted guideline for neuro-pathological grading that allows clinicians and scientists to coordinate treatment and research efforts across institutions and nations. Subsequent iterations (Kleihues et al., 1993; Kleihues and Cavenee, 2000; Louis et al., 2007) of this guide show the evolution in understanding of these diseases based upon improving technologies – for example, immunohistochemistry, magnetic resonance imaging (MRI), and tumor genomes – as well as newly defined, unique tumor types, e.g. extraventricular neurocytoma (Louis et al., 2007).

Primary CNS tumors are defined as those cancers of neuroepithelial origin within the brain and spinal cord, and currently have an overall incidence in the United States of 18.16 per 100,000 person-years (CBTRUS Report, 2009). This is not to be confused with brain metastases from other origins such
as lung or breast cancers. The most frequent singularly diagnosed type is meningioma, accounting for 33% of all primary CNS tumors. Fortunately, meningioma is most often a benign condition that can be cured with surgical intervention. Equally incident to meningiomas is a broad class known as the gliomas, which most commonly arise within the cranial vault. What is most striking about gliomas is that they currently account for 80% of all CNS malignancies – those cancers that are particularly aggressive, becoming terminal illnesses over time even with treatment. By far the most frequent glioma is glioblastoma multiforme (GBM), the biology of which is the focus of this work.

By WHO grading standards, GBM is regarded as Grade IV astrocytoma. It is a particularly insidious affliction that, after decades of research into the matter, still eludes efficacious treatment. On average in the United States, approximately 10,000 new diagnoses of GBM will occur every year, and of these fewer than half the individuals will survive an additional year. Less than five percent of those diagnosed with GBM will survive to two years (Adamson et al., 2009; Fisher et al., 2007). While malignant glioma accounts for less than two percent of adult cancers, it is compelling to discover better ways to treat and fight it. It debilitates its victims in staggeringly short course, with profound resistance to current
therapies, or otherwise making treatment difficult as it progressively assaults the organ of human being, personality, emotion, and thought.

I.2 Grading and Distribution of the Gliomas

Gliomas are so named because of their resemblances to CNS glial cells; thus they are generally distributed as ependymomas, oligodendrogliomas, astrocytomas, and tumors of mixed glial cytoarchitecture and other characteristics. It was originally assumed by many that gliomas possess these features because they are risen from late-stage glia by some carcinogenic event(s), but concrete epidemiological or etiological data were lacking. However, within this decade the identification of cancer stem cells has been described within these tumors, and their functions and origins are under investigation (Louis, 2006; Suvà et al., 2009).

Gliomas can be divided between low- and high-grade tumors based upon the WHO grades I-IV scale of malignancy. The lowest, grade I, denotes tumors that can be considered benign and only require surgical removal for curative effect. Most ependymomas and the rare pilocytic astrocytoma, fall into the Grade I category. When tumors present increased cellularity, indicative of more proliferation, and the beginnings of infiltration into normal parenchyma, they are classified as grade II; this is one
of two currently accepted descriptors for oligodendrogliomas, the other being grade III for more aggressive tumors, but there is substantial debate regarding the necessity for expanding oligodendroglioma categorization. As with grade I, astrocytomas rarely fit into the grade II grouping. There has been notable concern regarding the efficacy of radiologic and chemotherapeutic treatments for grade II conditions, as these modalities can elicit more discomforting symptoms with little to no benefit (Buckner et al., 2007). Interestingly, the lower grade tumors, especially ependymomas and pilocytic astrocytomas, are more common in more youthful populations.

The high-grade primary CNS tumors are split between grades III and IV. Tumors are staged at grade III when there is clear presentation of tumor cell invasion, anaplastic transformation (apparent de-differentiation or primitive cell types), and extensive proliferation, i.e. mitotic figures. The aggressive oligodendrogliomas and anaplastic astrocytomas are considered grade III. Diagnosis with a grade III neoplasm is usually a terminal condition; however grade III oligodendrogliomas have the highest extended survival rates notwithstanding substantial variability in median survival within histologic sub-types, e.g. 2-2.5 years for anaplastic astrocytoma and 3-5 years for anaplastic oligodendrogliomas (Buckner et al., 2007).
If a grade III tumor does not result in a terminal condition it will invariably progress to a grade IV disease. In the context of primary CNS tumors of the developed brain, i.e. excluding embryonal tumors and brain metastases, GBM is the only grade IV tumor. It is characterized by a fast course from diagnosis to untenable tumor growth, which presents within the tumor as core necrotic areas, peripheral endothelial hyperplasia, and inaccessible tumor cell infiltrate into normal host tissue. After treatment, a secondary lesion will almost always occur which, combined with other associated neurological sequelae, will lead to death. Median survival post-diagnosis with GBM is approximately one year (McKinney, 2004; Buckner et al., 2007).

It is clear that there is a vast array of histologically distinct tumor types, and that with the lower grade gliomas and meningiomas there is a promising outlook for patient survival and quality of life. Unfortunately, the worst of these cancers, GBM, is the second most common primary CNS diagnosis, regardless of malignancy. Furthermore, GBM accounts for a disproportionately high share of primary CNS malignancies, and has been on the rise since the extensive recording of such data, with current figures placing it at 20% of all CNS tumors and 54% of diagnosed gliomas (Figure 1.1).
Figure 1.1. Distributions of CNS Neoplasia. Panel A illustrates the distribution of all primary CNS tumors diagnosed in the United States for the period 2004-2005 (N=98,990). Meningioma is the single most common tumor yet is predominantly benign. However, 80% of primary CNS malignancies are classified as various gliomas. ‘Other’ tumors in Panel A are either unclassified histological sub-types or uncommon sub-types with lower incidence than can be graphically represented. Panel B illustrates the distribution for all diagnosed gliomas in the same period (N=32,279). Glioblastoma multiforme (GBM) is by far the most common at 54%. ‘Other astrocytoma’ includes astrocytic tumors with mixed characteristics, or cases with unique characteristics. ‘Other gliomas’ includes mixed gliomas or other rare neuroepithelialial tumors. This figure was constructed using data from the CBTRUS 2009 Report.
A. All Primary CNS Tumors Diagnosed 2004-2005 in the United States

B. All Gliomas Diagnosed 2004-2005 in the United States
I.3 Epidemiology and Current Standard of Care for GBM

The prediction of GBM remains difficult because its complete etiology remains unknown. Compounding this matter is the fact that GBM is heterogeneous and arises in two forms: one aforementioned from progression of a lower grade condition, termed ‘secondary’ GBM; and another noted as ‘primary’ which indicates the de novo presentation of the disease. Over 90% of GBM diagnoses are of de novo origin (Ohgaki and Kleihues, 2009). The clinical histories for secondary GBM range from 1.4 years, if progressed from a grade III condition, to 5.6 years whence progressed from grade II. Illustrative of its short course, upwards of 86% of primary GBM patients have a clinical history of less than six months; yet mean age at diagnosis with secondary GBM is profoundly lower than primary, 45 years versus 62 years respectively (Ohgaki and Kleihues 2007 & 2009). Despite these differences in formation and certain molecular heterogeneity described later in this work, GBM as a whole converges upon and elicits the same gross pathologies that make it difficult to treat: invasion, angiogenesis and resistance to apoptosis.

Recorded data do indicate that the incidence of GBM is on the rise every year. While the incidence of all primary CNS tumors is higher in females as indicated by a current male to female ratio of 1:1.22, the reverse is true when examining
gliomas and specifically GBM. Gliomas are significantly more common in men at a 1.43:1 ratio, and this carries through with GBM at a 1.62:1 ratio for the most current publicly available data (CBTRUS, 2009). Other recent studies analyzing specific population sets and slightly older epidemiological data suggest that this male to female ratio of GBM incidence could be as high as 3:1 for primary GBM (Adamson et al., 2009). Despite this gender disparity, the strongest risk factor for GBM remains age, with incidence rate rising to a peak between 75 and 84 years (CBTRUS, 2009; Figure 1.2).

Recently, cellular phone usage has been popularly suggested as a predisposition to GBM, having educed special Congressional panels investigating the matter in the United States (Hardell et al., 2007; Hardell and Carlberg, 2009). However, the reported correlations remain weak due to response bias, and are difficult to reconcile in the face of improved technology and the rate at which cellular phone usage reached virtual ubiquity over the past decades. Other environmental factors have been proposed such as exposure to N-nitroso compounds or second-hand smoke, but no causal link has been proven in any case (McKinney, 2004; Fisher et al., 2007). Fewer than 5% of gliomas show familial propensity and have been reported to occur coincident with inherited malignancy disorders such as neurofibromatosis and Li-Fraumeni syndrome; but less than 1% of such a population studied
Figure 1.2. Increasing Age as a Predisposition for GBM. The incidence rate of GBM increases exponentially with age to 65-74 years, and peaks at 75-84 years. This figure was constructed using data from the CBTRUS 2009 Report.
GBM Incidence by Age, 2004-2005
had GBM (Wrensch et al., 1997). The only known definitive, direct cause of GBM is exposure to ionizing radiation as a treatment for children with acute lymphoblastic leukemia (Neglia et al., 1991).

As mentioned previously, survival and quality of life prospects with GBM diagnosis are grim. Due to the elevated intracranial pressure caused by the rapidity of tumor growth, initial clinical indications will commonly begin with headaches, nausea, and other symptoms of general malaise that become progressively worse without alleviation; about one-third of victims will suffer seizures (Buckner et al., 2007). The general rule is that approximately 70% of adult tumors will occur supratentorially and as such when particularly eloquent areas are invaded, partial loss of motor and sensory systems will occur associated with the location of the intracranial mass. Up to one-fifth of patients, especially those suffering frontal lobe tumors, will also have profound alterations in emotional and mental status (Behin et al., 2003; Buckner et al., 2007).

With the combination of these symptoms the physician would suspect a brain tumor, and the best method to continue diagnosis is with the use of contrast enhanced (gadolinium injection) MRI. A halo-like appearance of hyper-intensity is observed with GBM; this corresponds to the proliferative periphery of the tumor.
Highly invasive tumor cells are found around and outside of the halo, and the hypo-intense center represents the necrotic tumor mass. It is important to note that the associated intracranial pressure will elicit vasogenic edema that can be detected through T2-weighted MRI. Eventually, removal of the reasonably accessible tumor mass and its histopathological analysis will confirm GBM with the presence of pseudopallisading cells around areas of necrosis, intense tumor associated angiogenesis, and the piling of tumor cell masses around circulating blood vessels (Holland EC, 2000; Brat et al., 2004; Kaur et al., 2005).

The current standard treatment for GBM begins with surgical de-bulking of accessible tumor mass soon after initial diagnosis, since left untreated the tumor can lead to death within three months. For the six weeks subsequent to surgery, the patient receives targeted fractionated irradiation at 2Gy/day, five times per week. Concomitant with radiation will be temozolomide chemotherapy at 75mg/m$^2$ body surface area/day, and for up to six months following radiation 150mg-200mg/m$^2$ body surface area five times per month. Temozolomide is a DNA alkylating agent, thus inducing the DNA damage apoptotic response in highly proliferative GBM cells (Stupp et al., 2005; Jeon et al., 2009). It has been proven to facilitate the extension of median survival time, and is preferred to other systemic chemotherapies, such as cisplatin and carmustine.
(BCNU), because of equivalent effects with milder off-target toxicity (Silvani et al., 2009).

Overall, one-year post-diagnosis GBM patient survival ranges from 17%-30% and two-year survival falls under 5% (Adamson et al., 2009). Surgery plus radiotherapy produces a median survival time of 12.1 months, and the inclusion of temozolomide only extends this to 14.6 months (Stupp et al., 2005). Furthermore, GBM that expresses O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) is unresponsive to temozolomide treatment, as this enzyme alleviates the primary DNA damaging mechanism of that drug (Jacinto and Esteller, 2007). To improve the efficacy of temozolomide usage, agent combinatorial clinical trials are underway that involve the focal administration of BCNU and/or the ablation of MGMT activity through the administration of inactivating substrate O\textsuperscript{6}-benzylguanine, which thus far have displayed significant promise (McGirt et al., 2009; Quinn et al., 2009). However, a meta-analysis of various agent clinical trials over three decades demonstrated that there is a two-year survival benefit associated with trial enrollment. This has been attributed to selection bias and better access to care (Stewart, 2002).

Ultimately, GBM patient care will focus on palliative management of symptoms. After standard treatment a second lesion will likely occur, and at a site distant from the removed
primary mass. These recurrent tumors clonally adapt to treatment paradigms, particularly when DNA damage is involved. Younger age, extent of resection, and higher Karnofsky Performance Scale score are the best prognoses for longer survival. Regardless, if the individual does not fall victim to the increasing intracranial pressure, assuaged to a limited extent by corticosteroid treatment, he will eventually succumb to the damaging systemic side effects of radio- and chemotherapies.

I.4 Molecular Characteristics of GBM

It is now widely recognized that GBM is heterogeneous in the molecular origins of its pathologies; as mentioned before there is rarely a case when a single specific carcinogenic event educes gliomagenesis. Rather this is likely an additive effect of multiple tumorigenic transformations that take place at the genomic level: either through the loss of tumor suppressor genes and function, or through the amplification, over-expression and activation of oncogenes (Gu et al., 2009).

However, there are some common features among GBMs. The most frequent of these, up to 80%, is loss of heterozygosity (LOH) of the long arm of chromosome 10 (10q), thus implying the presence of multiple tumor suppressor gene loci across that region (Rasheed et al., 2005; Lam-Himlin et al., 2006). While
some primary GBMs display complete LOH, there are rarely examples of homozygous deletion of all or parts of chromosome 10. Within primary GBM, one of the most well studied of these 10q tumor suppressors is phosphatase and tensin homolog (PTEN, also known as mutated in multiple advance cancers 1 ‘MMAC1’, Steck et al., 1997), which is at the 10q23.3 locus and is either LOH or mutated in over 30% of GBM (Knobbe and Reifenberger, 2003). As the first phosphatase identified as a tumor suppressor, PTEN has generated considerable interest. It is an apparent inhibitor of phosphoinositide 3-kinases (PI3K), which are cell-signaling transducers active at the plasmalemma. Normally, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP₂) to form the active phosphatidylinositol (3, 4, 5)-trisphosphate (PIP₃). This tri-phosphate is required for the downstream activation of the AKT-mammalian target of rapamycin (mTOR) axis via phosphoinositide-dependent kinases (PDK), ultimately inducing the transcription of anti-apoptotic, pro-survival proteins. In a non-neoplastic cell PTEN inhibits this pathway by de-phosphorylating PIP₃ back to the inactive PIP₂ (Simpson and Parsons, 2001). More recently PTEN dysfunction in GBM has been implicated in the resistance to apoptosis by protein stabilization of survival factors, i.e. mutant PTEN prevents poly-ubiquitination (Panner et al., 2009). However, the deletion or mutation status of PTEN offers no prognostic
significance in GBM, which indicates that there are other factors vital to the progression of the disease (Lam-Himlin et al., 2006).

One such factor, the study of which bloomed in the mid-1990’s because of its association in multiple cancers, is p53. With its encoding gene (TP53) located on the short arm of chromosome 17, mutation of p53 is more incident than its LOH in GBM. In a healthy cell p53 protein stability is low, because Mdm2 binds to and facilitates poly-ubiquitination of p53, thereby causing its proteasomal degradation. When a normal cell is exposed to a plethora of stressful stimuli, particularly the induction of DNA damage, Mdm2 dysfunction and p53 stabilization are coincident. This activation of p53 leads to the expression of cell cycle arrest proteins, especially p21 and halting of the cell cycle at the G1/S transition, to allow the stimulation of DNA repair or apoptosis (Bögler et al., 1995; Ohgaki and Kleihues, 2007). In effect, p53 is a genome-stress sensitive tumor suppressor. Over 30% of GBMs have been found to express mutant p53, most of them secondary (Ohgaki et al., 2004). While mutation of p53 will often cause a loss of function, thereby allowing tumor cell growth, gain-of-function mutations have also been indicated (Bögler et al., 1995; Oren and Rotter, 2010). This is a likely factor confusing whether or not p53 status is a valuable prognostic feature and therapeutic target. Indeed, one
major study found that TP53 mutation is favorable to survival (Schmidt et al., 2002), yet others have reported the converse or insignificance (Simmons et al., 2001; Ohgaki et al., 2004).

Over-expression and amplification of certain genes and proteins are also fundamental molecular components of GBM. The most richly described of these factors is Endothelial Growth Factor Receptor (EGFR), a transmembrane receptor tyrosine kinase that is a member of the ErbB family (ErbB1). Multiple growth factors can activate EGFR including: EGF, transforming growth factor(TGF)-α, betacellulin and epiregulin. Once one of these ligands is bound, the EGFR monomer can either homodimerize or form a heterodimer with p185\textsuperscript{her2/neu}(ErbB2). This facilitates the autophosphorylation of specific tyrosine residues on the C-terminal end of the EGFR molecules. The pattern of phosphotyrosines serves as an activation anchor for other kinases with Src homology2 (SH2) domains, specifically PI3K and mitogen activated protein kinases (MAPK), which transduce pro-migratory, proliferative, and anti-apoptotic cellular signals (Zhang et al., 2007).

Amplification of EGFR, either wild type or mutant, is observed in approximately 40% of GBM cases, yet interestingly is not seen in patients less than 25-years old (Ohgaki et al., 2004). Seven predominant, active EGFR mutants have been identified, which are for the most part caused by in-frame
deletions of the EGFR gene. By far the most common in GBM is named EGFRvIII, found in 50% of amplified tumors (Schwechheimer et al., 1995; Rasheed et al., 1999; Adamson et al., 2009). This particular mutant is created by an 801-bp in-frame deletion of exons two through seven, and is constitutively active, i.e. does not require ligand (Biernat et al., 2004). Since mutant EGFR amplification is unique to the GBM site, and is thought to play a vital role in mediating GBM pathology, it is a promising therapeutic target. Indeed, in a trial with non-small-cell lung carcinoma (NSCLC) patients (another cancer with unique EGFR amplification), a specific inhibitor, gefitinib, showed efficacy (Fukuoka et al., 2003). However similar results with GBM have been lacking: while the therapy is tolerable, no objective tumor responses were seen with significance across glioma populations (Rich et al., 2003; Reardon et al., 2010). This is likely due to at least two important details. First unlike NSCLC, mutations in the kinase domain of EGFR are rare if non-existent in glioma (Marie et al., 2005). Second, it was later discovered, in the context of EGFRvIII inhibitors alone (Rich et al., 2003), that while EGFRvIII is the most common mutant in GBM, its predominance over concomitantly amplified wild type EGFR is a rare event (<10%); thus the inhibition of EGFRvIII is easily circumvented by tumor cells (Biernat et al., 2004). An intriguing subsequent development was the observation that GBM
patients with tumor EGFRvIII and PTEN co-expression responded favorably to anti-EGFR treatments (Mellinghoff et al., 2005). The latter study underscores the evolving concept of the importance of multi-modal, patient specific, molecular targeted therapeutics.

A great deal of excitement, as well as debate, is currently centered upon the inhibition of vascular endothelial growth factor (VEGF)-A. Since the standard treatment paradigm for GBM is limited in scope and efficacy, therapeutics useful in unrelated cancer conditions are frequently trialed with GBM. Bevacizumab is a humanized monoclonal antibody raised against VEGF-A, which has offered significant recourse against colorectal (Hurwitz et al., 2004), breast (Miller et al., 2005), renal (Escudier et al., 2007), and lung (Herbst et al., 2007) cancers. Two recurrent GBM clinical trials testing bevacizumab showed substantial improvements in tumor response and six month progression free survival (Cohen et al., 2009; Friedman et al., 2009). Based upon these findings, where the endpoints were assessed by independent radiologist review of MRIs, the USFDA fast-tracked the approval of bevacizumab therapy in recurrent GBMs non-responsive to other treatments, and its use in newly diagnosed tumors is currently under trial. Despite these promising data, credible concerns have been voiced regarding this particular therapy. Unlike the tightly controlled normal
blood-brain barrier, GBM vasculature is characteristically leaky, and this leakiness is what elicits the classic T1-weighted GBM images. Inhibition of VEGF-A potentially normalizes and limits this leakiness, therefore offering an increased probability for false negative assessments on contrast enhanced MRI review. When evaluated at Phase III this might become more apparent, but other indicators within the endpoints used for the Phase II trials corroborate favorable effect. Another concern is the fact that the normalization of the tumor-blood barrier could reduce the efficacy of systemically delivered chemotherapeutics that rely on the leaky tumor vasculature. Finally available anti-angiogenic therapies, such as bevacizumab, are inert with regard to the invasive facet of GBM pathology, and have even been hypothesized to enhance invasion (Verhoeff et al., 2009; Lucio-Eterovic et al., 2009).

While there are some molecular propensities among GBMs, none of those that are intensely investigated are present in 100% of cases, or are entirely unique in the context of the local host environment. In cases where there is some exclusivity, e.g. EGFRvIII, the tumor cells because of their heterogeneous molecular profile, will often adapt to treatment efforts through some other mechanism, e.g. switching from PI3K dependence to MAPK (Albert et al., 2009). The continued investigation of molecular targeted therapeutics is desperately
needed and many are currently underway, with over 200 clinical trials ongoing in the United States as of 2010. Some of these are outlined in Table 1.1 (information provided by the National Institutes of Health). The idea of multi-modal therapies tailored to the molecular profiles of individual tumors is becoming more favored for particularly aggressive cancers like GBM. However it is still important to discover and investigate potential targets that can address multiple GBM pathologies (invasion, angiogenesis, and resistance to apoptosis/treatment) at the same time. Based upon this goal, one particular protein known as matrix metalloproteinase-1 (MMP-1) is the focus of this work.

I.5 Structure of the Matrix Metalloproteinases

To date twenty-four distinct proteins are included in a family of zinc-dependent endopeptidases known as the matrix metalloproteinases (Table 1.2). These enzymes are well known for their abilities to degrade a variety of extracellular matrix (ECM) constituents, the first one discovered having been characterized as an effective interstitial collagenase (MMP-1; Gross and Lapiere 1962). Over the four decades of study into these enzymes it has been discovered that MMPs are far more flexible in other activities than ECM degradation and remodeling. The MMPs are also potent protease modulators of
Table 1.1. Agents in Multiple Clinical Trials for GBM. There is a diversity of different drug types and targets, though there is a current focus on multiple targets along the PI3K-Akt axis. HDAC: histone deacetylases; MGMT: O\textsuperscript{6}-methylguanine-DNA methyltransferase. Not included: AP12009 (small molecule inhibitor of TGF-\(\beta\)) and TP-38 (TGF-\(\alpha/pseudomonas\) exotoxin chimera) were recently concluded in several studies.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Type/Description</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>tipifarnib</td>
<td>quinolinone derivative</td>
<td>farnesyl transferase</td>
</tr>
<tr>
<td>cilengitide</td>
<td>cyclic peptide</td>
<td>α4β5 integrin</td>
</tr>
<tr>
<td>IL13-PE38QQR</td>
<td>chimeric pseudomonas toxin</td>
<td>IL-13 Receptor</td>
</tr>
<tr>
<td>bevacizumab</td>
<td>monoclonal antibody</td>
<td>VEGF-A</td>
</tr>
<tr>
<td>dasatinib</td>
<td>small molecule</td>
<td>Src</td>
</tr>
<tr>
<td>Erlotinib/gefitinib</td>
<td>quinazoline derivative</td>
<td>EGFR</td>
</tr>
<tr>
<td>perifosine</td>
<td>alkyl phospholipid</td>
<td>Akt</td>
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<td>sirolimus</td>
<td>macrolide</td>
<td>mTOR</td>
</tr>
<tr>
<td>everolimus</td>
<td>Sirolimus derivative</td>
<td>mTOR</td>
</tr>
<tr>
<td>vorinostat</td>
<td>polar-planar enzyme inhibitor</td>
<td>HDAC</td>
</tr>
<tr>
<td>O(6)-benzylguanine</td>
<td>Guanine analog</td>
<td>MGMT</td>
</tr>
</tbody>
</table>
bioactive molecules and chemotactic positioning. They are integral enzymes in processes of wound healing, cell differentiation, development, inflammation, proliferation, apoptosis and migration (McCawley and Matrisian, 2001; Brinckerhoff and Matrisian, 2002; Chakraborti et al., 2003). An assortment of MMPs has been observed in excess throughout varying loci of gliomas which are hypothesized to be integral components of microenvironment conducive to progression, underlining their importance in pathological states (Fillmore et al., 2001; VanMeter et al., 2001).

Individual MMPs are often referred to by substrate specificity (collagenases, gelatinases, stromelysins, matrilysins) but the advent of evidence showing that these characteristics are overlapping throughout the MMP family led to their recognition by protein structure as well (Figure 1.3A). All MMPs consist of at least three common domains: a signal peptide that directs its translation on the endoplasmic reticulum, a pro-domain that maintains the latency of the enzyme via the binding of a conserved cysteine to the requisite zinc atom, and a catalytic domain which is the enzyme functioning portion that also binds the zinc. Only two of the MMPs (7 & 26) consist of just the minimal domains. Ten MMPs (1, 3, 8, 10, 12, 13, 19, 20, 27 & 28) have the core domains along with a hemopexin domain that is required for protein-protein
Table 1.2. **The Matrix Metalloproteinases.** There are currently twenty-four recognized MMPs. *MMP-18* is a *Xenopus* collagenase not found in humans. \(^\text{a}\)MMP-23 is duplicated in human lineage and sometimes referred to as either MMP-23A or MMP-23B (Puente et al., 2005). MMP-19 is often referred to as RASI (rheumatoid arthritis synovial inflammation) because of its location (Pasternack and Aspenberg, 2009), whilst ‘stromelysin-4’ is an oft-used misnomer.
<table>
<thead>
<tr>
<th>Enzyme Type</th>
<th>MMP ID</th>
<th>Other Name(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>MMP-1</td>
<td>Interstitial Collagenase; Collagenase-1</td>
</tr>
<tr>
<td></td>
<td>MMP-8</td>
<td>Neutrophil Collagenase; Collagenase-2</td>
</tr>
<tr>
<td></td>
<td>MMP-13</td>
<td>Collagenase-3</td>
</tr>
<tr>
<td></td>
<td>MMP-18*</td>
<td>Collagenase-4</td>
</tr>
<tr>
<td>Gelatinases</td>
<td>MMP-2</td>
<td>72kDa Gelatinase; Gelatinase- A</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>92kDa Gelatinase; Gelatinase-B</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-3</td>
<td>Transin; Stromelysin-1</td>
</tr>
<tr>
<td></td>
<td>MMP-10</td>
<td>Stromelysin-2</td>
</tr>
<tr>
<td></td>
<td>MMP-11</td>
<td>Stromelysin-3</td>
</tr>
<tr>
<td>Matrilysins</td>
<td>MMP-7</td>
<td>Matrilysin-1</td>
</tr>
<tr>
<td></td>
<td>MMP-26</td>
<td>Matrilysin-2</td>
</tr>
<tr>
<td>Membrane-Type (Transmembrane)</td>
<td>MMP-14</td>
<td>MT1-MMP</td>
</tr>
<tr>
<td></td>
<td>MMP-15</td>
<td>MT2-MMP</td>
</tr>
<tr>
<td></td>
<td>MMP-16</td>
<td>MT3-MMP</td>
</tr>
<tr>
<td></td>
<td>MMP-24</td>
<td>MT5-MMP</td>
</tr>
<tr>
<td>Membrane-Type (GPI Anchored)</td>
<td>MMP-17</td>
<td>MT4-MMP</td>
</tr>
<tr>
<td></td>
<td>MMP-25</td>
<td>MT6-MMP</td>
</tr>
<tr>
<td>Other</td>
<td>MMP-12</td>
<td>Macrophage Elastase; Metalloelastase</td>
</tr>
<tr>
<td></td>
<td>MMP-19</td>
<td>Stromelysin-4; RASI</td>
</tr>
<tr>
<td></td>
<td>MMP-20</td>
<td>Enamelysin</td>
</tr>
<tr>
<td></td>
<td>MMP-21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-23</td>
<td>Cysteine Array(CA)-MMP</td>
</tr>
<tr>
<td></td>
<td>MMP-27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-28</td>
<td>Epilysin</td>
</tr>
</tbody>
</table>
interaction and its linker, a hinge domain, which can also impart specificity. With the exception of the cysteine array MMP (23) all the other MMPs have the five aforementioned domains with further additions. The classic gelatinases, MMP-2 and -9, have fibronectin-like repeats in the catalytic domain required for substrate binding, and MMP-9 also has a type V collagen-like domain. In addition, MMP-11 and the membrane-type MMPs (14, 15, 16, 17, 24, 25) have furin cleavage sites in the pro-domain that are important for pro-domain clearance and enzyme activation (MMP-23 has a furin cleavage site too). The membrane-type MMPs are further subdivided based on how they are associated with the plasmalemma: either through a single transmembrane domain (MMP-14, 15, 16 & 24) or by a glycosyl phosphatidylinositol (GPI) anchor (MMP-17 & 25; McCawley and Matrisian, 2001; Nagase et al., 2006).

I.6 Regulation of the Matrix Metalloproteinases

Matrix metalloproteinases are initially synthesized as zymogens, latent enzymes that require some other event for activation. The thiol of a conserved cysteine residue (PRCGXP) in the pro-domain maintains the zymogen by binding a zinc atom that is chelated by conserved histidines (HEXXHXXGXXH) in the catalytic domain. This effectively blocks the active site with the pro-domain and prevents water molecules from an interaction that allows proteolytic activity. The enzyme is activated following
**Figure 1.3. Domain Schemes of the MMPs, and the Cysteine Switch.**

A. The various MMPs categorized by similar domain construction (the arrow denotes that the pro-domain associates with the catalytic domain when the enzyme is latent); MMP-7 and -26 represent the minimal domain set required to be classified as a MMP: signal peptide, pro-domain with conserved sequence PRGCXPD, and catalytic domain with the conserved sequence HEXXHXXGXXH.

B. Diagram of the cysteine switch where under the latent zymogen condition the pro-domain (red) coordinates with the catalytic site zinc atom (chelated by the three conserved histidine residues, H in above catalytic domain sequence) via the conserved cysteine thiol (C in conserved pro-domain sequence above). When the pro-domain is attached it prevents hydrolysis by obstructing the access of water. Once the pro-domain is removed, water is free to enter and enzyme activity may commence.
B. \( \text{H}_2\text{O} \)

Pro-domain cleavage event

\( \text{H}_2\text{O} \)
displacement of the cysteine-zinc bond or altogether removal of the pro-domain; this mechanism is referred to as the ‘cysteine switch’ (Figure 1.3B; Van Wart and Bikedal-Hansen, 1990). MMP activation is multi-factorial by way of direct methods or by other MMPs. For example, plasmin can activate several MMPs including MMP-3; MMP-3 in turn can activate MMP-1, -7, -8, -9 and -13 (Chakraborti et al., 2003; Pasternak and Aspenberg, 2009). Interestingly, the activation of MMP-2 can be facilitated by TIMP-2, a traditional MMP inhibitor. Membrane Type-1 MMP (MMP-14) associates with TIMP-2 (binds to the MT-1 catalytic site) and together they localize at the cell membrane. Then TIMP-2 also interacts with pro-MMP-2 bringing it into close association with another free MT-1 (the MT-1 is understood as a dimer by some), which cleaves the pro-domain and switches it to the active MMP-2 (Hernandez-Barrantes et al., 2000; Wang et al., 2000).

Four groups of physiological MMP inhibitors have been identified. One large inhibitor (772kDa), α2-macroglobulin, operates primarily in blood circulation by a bait-trap mechanism. MMPs recognize small portions of α2-macroglobulin and cleave them facilitating a conformational change in the inhibitor that engulfs the MMP. The complex binds to its receptor (LDL-RP) and is internalized to the cell where degradation takes place. Another inhibitor, reversion inducing
cysteine-rich protein with Kazal motifs (RECK), is membrane associated through a GPI anchor and thus far has been shown to inhibit secretion and substrate binding of MMP-2, -9, and -14. Lower than normal expression profiles of RECK have been observed in tumor cell lines, suggesting a partial loss of control over some MMPs. Interestingly, RECK knockout animals die pre-natal which also insinuates that there may be more biologically vital activities involved with RECK than MMPs alone (Oh et al., 2001; Baker et al., 2002). A third set of inhibitors is the secreted tissue inhibitors of metalloproteinases (TIMP), a class that consists of four distinguishable proteins. The TIMPs are known to broadly inhibit active MMPs by binding the active site similarly to normal substrates. However, the efficacy of MT-MMP inhibition by TIMP-1 is lacking, and the TIMPs also have well known associations with pro-MMPs such as the requirement of TIMP-2 for proMMP-2 activation mentioned previously. Further unconventional effects of the TIMPs have been reported for features that impact tumor progression such as apoptosis, proliferation, adhesion and angiogenesis. The observations of the extensive roles of TIMPs have largely been cell/tissue specific and many questions still remain regarding this ever-growing topic. The fourth set of inhibitors are the TIMP-like molecules which includes a group of proposed, based on binding sequence similarity to TIMPs, and experimentally derived
inhibitors such as the type I collagen C-proteinase enhancer (PCPE) protein (Baker et al., 2002).

The MMPs are also transcriptionally regulated, with a great deal of similarity among MMP gene promoters. Their promoters can be classified into three general groups (Yan and Boyd, 2007). The first classification is the largest (MMP-1, -7, -9, -10, -12, -13, -19, -26) and denotes the most complex MMP promoters that share in common a TATA box and an activator protein (AP) -1 consensus sequence approximately 30bp and 70bp respectively upstream of the transcription start site. Most of these promoters also have polyomavirus enhancer activator 3 (PEA3) sites that act in concert with the proximal AP-1 and sometimes other distal AP-1 loci (Benbow and Brinckerhoff 1997). The second promoter group (MMP-8, -11, -21) only has the proximal TATA box in common, and at least to date have comparably straightforward structures. The final group (MMP-2, -14, -28) has neither the proximal TATA box nor AP-1 site (Yan et al., 2007). Unlike the other MMPs, this group is constitutively expressed in certain normal tissues (Chakraborti et al., 2003). There are tissue specificities, such as with MMP-28 being limited to developing germ cells and MMP-13 being restricted to osteoblasts. However, the details behind this specificity remain largely unknown and probably depend on the
restricted expression of other transcription factors not yet identified to act upon MMP promoters (Yan and Boyd, 2007).

The presence of AP-1 sites in the majority of MMP promoters implies sensitivity to transduced extracellular signals, primarily through the MAPK pathway. This includes stimulation through growth factors (EGF, VEGF, HGF, bFGF), cytokines (INF, IL-1β, TNF-α, TGF-β), and ECM sensation by the integrins (DeMali et al., 2003; McCready et al., 2005; Yan and Boyd, 2007). Furthermore MMP-1, -3, -9, and -11 are profoundly induced by nuclear factor-κB (NF-κB) activity. This is readily apparent in MMP-9 as its promoter harbors a traditional NF-κB consensus, whereas the others do not (Yan et al., 2001; Vincenti and Brinckerhoff, 2002). The reasons for this are currently under investigation and likely involve either non-standard NF-κB binding sites in MMP promoters or the transactivation of other transcription factors to which these MMPs are downstream.

Promoter hypermethylation, which is normally targeted to CpG islands, has also been linked to repression of MMP-3 and -9 transcription (despite their lack of CpG islands), but not MMP-1 and -2 (Chicoine et al., 2002; Couillard et al., 2006). Another point of control involves chromatin structure, specifically through the acetylation and phosphorylation of histones. Unfortunately, the epigenetic aspects of MMP regulation are poorly understood and require substantial work to establish any
further conclusions. The stability of mRNA is another important step in the regulation of MMP expression. The stabilization of MMP-2, -9, and -13 mRNAs by TFG-β has been well established. At least with the MMP-9 transcript, specific areas of the 3′-untranslated region (UTR) have been identified that interact with HuR, an mRNA stabilization factor (Yan and Boyd, 2007).

Finally, the presence of various promoter polymorphisms has been detailed in several of the MMPs. Changes in base pair sequence can directly influence the binding affinities for transcription factors. Transitions from cytosine (C) to thymine (T) at -1306 and guanine (G) to adenine (A) 1576bp upstream in the MMP-2 promoter reduce gene transcription (Price et al., 2001; Harendza et al., 2003). An A to G substitution in the MMP-12 promoter has a similar effect, however a C to T change in the MMP-9 promoter (-1562) increases expression (Jormsjö et al., 2000; Zhang et al., 1999). The MMP-3 promoter is susceptible to the insertion of an extra A at -1171, termed the 6A allele. This allele has a propensity to bind inhibitory transcription factors over the normal 5A allele (Ye et al., 1999). Matrix metalloproteinase-1 also harbors an insertion, an extra G 1607bp upstream of the start site. This 2G allele introduces an Ets-1 binding site that acts in concert with a distal AP-1 locus, and as yet other unidentified transcription factors, to educe a ras
responsive element (RRE) that greatly increases promoter activity (Rutter et al., 1998; McCready et al., 2005).

I.7 Matrix Metalloproteinases in Brain Cancer

The gelatinases have been the most heavily studied MMPs with regard to all forms of cancer, especially glioma (Levičar et al., 2003; Björklund and Koivunen, 2005). It has been reported that MMP-2 and -9 expression is correlated with increasing glioma grade and tumor cell invasion in vitro (Sawaya et al., 1996; Uhm et al. 1996; Rooprai et al., 1998). As mentioned earlier, the MMP-2 promoter is constitutively active and non-responsive to growth factor stimulation. Indeed, the protein is also constitutively translated and detectable in its pro-form. However, its activity is proffered in a non-specific, unpredictable manner (Vince et al., 1999). More recent studies have pursued the inhibition of MMP-2 in glioblastoma cultures and in vivo modeling to show a reduction in invasion and angiogenesis (Kargiotis et al., 2008). However, later work demonstrated that the same inhibition of MMP-2 results in a further increase in EGFR in already amplified GBM cell lines and xenografts (Gondi et al., 2009). This could lead to a pro-tumor effect that exacerbates GBM pathologies, further diminishing its prospect as a good therapeutic target. On the other hand, MMP-9
is inducible by various forms of growth factor signaling that are aberrantly controlled in GBM (Levičar et al., 2003; Yan and Boyd 2007). Past studies have reported that MMP-9 inhibition reduces GBM cell invasion (Kondraganti et al., 2000). This is rather contrary to observations that MMP-9 is predominant in benign tumors, e.g. meningioma (Nakagawa et al., 1994). Nevertheless, MMP-9 is detectable in some GBMs to a varying degree, yet this expression is neither specific to tumor cells nor correlates with proliferative tumor zones (Vince et al., 1999). Later work demonstrated that the MMP-9 hemopexin domain actually inhibits GBM growth and related angiogenesis (Ezhilarasan et al., 2008). Thus the importance of MMP-9 in facilitating GBM pathology has progressively become more questionable.

The unique MT-MMPs have also been the focus of some study in the context of GBM. Membrane type-1 MMP was found to be differentially stimulated in vitro in various malignant glioma cell lines, but only following treatment with concanavalin A (ConA, Yamamoto et al., 1996). It was noted in the same study that while ConA treatment did not stimulate MMP-2 expression (and should not have since MMP-2 is non-responsive to mitogenic signal transduction), it did induce gelatinase activity concomitant with MT1-MMP production. These observations were recorded before the seminal studies demonstrating that MT1-MMP
and TIMP-2 are critical components of MMP-2 activation. A model was proposed whereby MT1-MMP is one of the factors of malignant glioma amplification, and it serves as a molecular bridge facilitating MMP-2 activity at the invasive membrane front of GBM cells (Fillmore et al., 2001). Subsequent work closely examined all of the MT-MMPs with respect to common GBM pathology in vitro, namely EGFR signal amplification. While the mRNA levels of all MT-MMPs were higher in cultures with EGFR activation, MT1-MMP was by far the most prevalent with upwards of a 4-fold induction. Furthermore, MT1-MMP was the only member of the group that had significantly increased protein levels. Levels of MMP-2 subsequent to treatment did not change, however activity did increase. The use of EGFR and PI3K inhibitors prevented this MT1-MMP expression, and in vitro invasion (Van Meter et al., 2004). Therefore it was found that at least in vitro MT1-MMP is an important mediator of EGFR signaling related invasion.

Matrilysin (MMP-7) has also been examined in patient GBM samples, where it was found to be predominantly expressed in hypercellular regions. It is hypothesized to be more important for initial tumor establishment and growth rather than invasion or metastasis – its precise role in malignant glioma remains to be studied (Vince et al., 1999). The expression of MMP-3 was reported in squamous cell carcinoma invasive processes and
subsequently examined in malignant glioma cell cultures, where its expression was found and could be induced by heat shock (Matsuzawa et al., 1996). However in the same work using fluorescence in situ hybridization and later studies investigating patient GBM samples, there is little to no MMP-3 expression (Vince et al., 1999).

The MMP-1 promoter work of the Brinckerhoff laboratory stimulated a new research front with regard to neoplastic processes (Benbow and Brinckerhoff, 1997; Rutter et al., 1998). Indeed, MMP-1 was reported to influence tumor cell invasion in a melanoma and eventually breast setting (Benbow et al., 1999). An early study suggested that in vitro, MMP-1 might be expressed by at least one glioma cell line (Nakano et al., 1995). The work of McCready and colleagues (2005) established that in fact MMP-1 is expressed at the mRNA, and importantly the mature protein level in patient GBM samples. Their in vitro work did show that the 2G promoter enhanced expression, and in patient samples the 2G allele was more prevalent. What is most profound from this study is that MMP-1 expression was exclusive to the tumors; MMP-1 was not detected in any normal brain controls. A recent study has confirmed that MMP-1 mRNA is increased in a larger set of GBM patient biopsies and in some cases with an increased gene copy number; and immunohistochemical data have
revealed pronounced MMP-1 expression throughout human tumors (Hodgson et al., 2009; Anand et al., under review).

This accumulating evidence, especially the uniqueness of MMP-1 in the context of the local host environment, presents this enzyme as a promising target for therapy. The lack of knowledge on MMP-1 in primary CNS neoplasia is largely due in part to the concept that it is a collagenase, and its traditional substrates are often absent in the CNS milieu. However, the idea of traditional substrates requires reconsideration owing to concrete data demonstrating that MMP-1 is an important activator of other MMPs, a potent liberator of bio-active molecules from the ECM such as FGF and insulin-like growth factor (IGF), and even the effector of the pro-angiogenic G-protein coupled receptor (GPCR) protease activated receptor-1 (PAR-1; McCawley and Matrisian, 2001; Blackburn and Brinckerhoff, 2008). As such factors that can induce the expression of MMP-1 in GBM cells and the functional consequences are under investigation. Upon examining the past work of others, one finds that an attractive regulatory target of MMP-1 expression is a small bio-active molecule, nitric oxide (NO), produced by another set of enzymes, the NO synthases (NOS), which are in part induced in high grade glioma. The following sections introduce NO and its relevance to brain cancer.
I.8 Nitric Oxide and the Nitric Oxide Synthases

The 1998 Nobel Prize for Physiology or Medicine was shared among Robert Furchgott, Ferid Murad, and Louis Ignarro for their contributions to characterizing NO, formerly known as endothelial-derived relaxing factor (EDRF), as it relates to blood circulation and the stimulation/maintenance of vasodilation. The abundance of information on the 30Da molecule is attributable to many discoveries into its biological roles over nearly thirty years. Originally known as an atmospheric pollutant, by 1987 research had shown that NO is an important physiological mediator of blood flow, specifically endothelial cell dependent vasorelaxation (Nathan and Xie, 1994; Furchgott, 1999). In 1988 Moncada and colleagues began publishing findings that the conversion of L-arginine to L-citrulline by an oxygenase is the primary mechanism of NO production in living systems (Palmer et al., 1988). Soon afterward this enzyme was characterized as what is presently known as nitric oxide synthase (NOS) and was classified in two distinct categories: constitutive (cNOS) which includes sub forms neuronal (nNOS/NOS1) and endothelial (eNOS/NOS3); and inducible (iNOS/NOS2).

To date it is known that NO is vital to many different physiologic aspects including immune functions, neurotransmission, blood pressure, and development in multiple
systems. Nitric oxide signaling to the latter three processes is regarded as results of low level NO pulse production via cNOS (nNOS and eNOS) which relies on intracellular calcium (Ca\(^{2+}\)) flux. Extensive diversity exists in the source stimuli of intracellular Ca\(^{2+}\) increase, e.g. hormones, growth factors and stress, allowing calmodulin (CaM) formation and binding with cNOS. This binding leads to the production, by NOS, of NO and L-citrulline from L-arginine and oxygen in the presence of other cofactors, e.g. NADPH, FAD, FMN, heme and tetrahydrobiopterin. Once NO is made, the classical method of signaling is through the NO-cGMP pathway. Cytosolic NO induces a conformational change and activates soluble guanylate cyclase (sGC) thus facilitating the conversion of GTP to cGMP. Cyclic GMP acts broadly as a second messenger and can influence ion channels and protein kinase activation, both of which imply significant downstream effects (Shinoda and Whittle, 2001; Fukumura et al., 2006).

Unlike its counterparts, iNOS is neither found readily in action throughout the body, nor is it as wholly dependent on Ca\(^{2+}\). The normal function of the enzyme is the production of NO on a massive, sustainable scale (\(\mu M\)) compared to the constitutive isoforms (nM). Induction of iNOS expression by such entities as macrophages, Kupffer cells, neutrophils, mast cells, microglia and astrocytes is one of many responses to cytokines such as
IFN-γ, TNF-α, and IL-1β as well as bacterial endotoxins e.g. lipopolysaccharide (LPS). The resultant cGMP independent effects are DNA damage (deamination) induced cytostasis (interruption of the cell cycle) and cytotoxicity, inhibition of DNA repair mechanisms, depletion of iron, and inhibition of the mitochondrial electron transport chain through NO and related reactive nitrogen species (nitrite, nitrate, peroxynitrite, etc.). Conversely, other immunomodulatory peptides like TGF-β, IL-4 and IL-10 can inhibit iNOS by message interruption and decay. Therefore, iNOS in a normal physiological context is commonly seen as the enzyme responsible for NO production as a host immune response-defense (Lala and Chakraborty, 2001; Fukumura et al., 2006).

Despite these altogether helpful roles, NO has also arisen as a player in pathological processes of several diseases, notably cancer. Nitric oxide and peroxynitrite can act as mutagens and have the ability to modify cell cycle control mechanisms such as the mutation and/or accumulation of standard tumor suppressors like p53 and the activation of other genes. Furthermore, the normal signaling mechanisms introduced above also hold sway over pathways involved in proliferation, apoptosis and cell-matrix/environment interactions, and potentially make the difference between tumor regression versus
neoplastic transformation, progression and invasion (Choi et al., 2001; Fukumura et al., 2006; Lam-Himlin et al., 2006).

I.9 Nitric Oxide in Brain Cancer

The first studies focused on NOS in brain tumors were by Cobbs and colleagues (1995). Immunohistochemistry using antibodies directed against all three isoforms of NOS as well as NADPHd staining (a marker of NOS activity, recall that NADPH is a cofactor of the enzymes) and immunoblot analysis of human brain tumor homogenates and normal brain for control were compared. They found that both nNOS and eNOS were grossly detectable in high-grade gliomas (grades III and IV) as opposed to lower grade and normal adult brain. Endothelial NOS was particularly concentrated in the vascular endothelial cells of the tumor vasculature. The inducible NOS was not detectable at large in most of the samples evaluated, aside from very faint immunohistochemical detection in GBM and anaplastic (grade III) astrocytomas. NOS activity was higher in all the tumors examined versus control. This work offered a couple interesting conclusions: presentation of the induction of cNOS in brain tumors, which was formerly believed to be non-inducible; and the localization of NOS and NO activity to tumors, especially vascular foci. The authors posit that NO is responsible for the pathology of tumor neovascularization, therefore subsequent progression.
Bakshi and coworkers (1998) offered the first evidence of NOS expression in peritumoral, edematous regions in the brain. The investigators were able to confirm earlier findings that cNOS was over-expressed in tumor cells, specifically surrounding vasculature, and that iNOS was barely present. Spatially, expression was markedly higher in tumor cores and dissipated with distance. They offered other novel insight with the observations: that in positive cells NOS was localized to the cytoplasm and nucleus, more so to the latter; that nNOS was remarkably intense in normal glial cells associated with a few tumors; and the presence of iNOS positive immune cells, apparently of monocytic lineage, that were invading a number of tumors beyond blood vessels. Work soon after this by another group correlated eNOS immunohistochemical labeling in the vasculature of astrocytic tumors with grade; labeling became more intense with anaplastic astrocytoma and GBM (Iwata et al., 1999).

What emerges not just in glioma but cancer conditions in general, is that there is a reorganization of NO activity with increasing degrees of malignancy; table 1.3 summarizes some of the key reports pertaining to primary CNS tumors. It is clear that cNOS expression is correlative with high-grade gliomas, thereby overlapping with MMP-1 expression. With regard to NO-influenced MMP-1 expression there have been at least two
Table 1.3. Nitric Oxide Synthase studies using patient glioma samples
<table>
<thead>
<tr>
<th>Study</th>
<th>cNOS Observations</th>
<th>iNOS observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobbs et al. 1995</td>
<td>Both increased with grade</td>
<td>Rarely detectable</td>
</tr>
<tr>
<td>Bakshi et al. 1998</td>
<td>Intense cNOS immunoreactivity associated with GBM</td>
<td>Not detectable in GBM</td>
</tr>
<tr>
<td>Iwata et al. 1999</td>
<td>eNOS immunoreactivity increased with grade and intense in GBM</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Ludwig et al. 2000</td>
<td>cNOS most detectable in GBM and associated endothelial cells, eNOS correlated with VEGF-R expression</td>
<td>Low Detection, primarily immune cells in nearby vasculature</td>
</tr>
<tr>
<td>Kao et al. 2003</td>
<td>Not assessed</td>
<td>Greater in glioma, particularly lower grades and in apoptotic areas</td>
</tr>
<tr>
<td>Broholm et al. 2003</td>
<td>nNOS increased with grade, eNOS mostly in endothelial cells</td>
<td>Sporadic detection in all tumors</td>
</tr>
<tr>
<td>Erdamar et al. 2006</td>
<td>Overexpression of eNOS and VEGF correlated with increasing grade. nNOS not assessed</td>
<td>Not assessed</td>
</tr>
</tbody>
</table>
reports. Yoshida *et al.* (2001) observed an induction of MMP-1 mRNA in cervical fibroblasts treated with DETA-NO and SNP (NO donor molecules). Ishii and coworkers (2002) demonstrated in human melanoma cell lines an increase of MMP-1 mRNA after exposure to SNAP (another NO donor) and transient transfection of an active iNOS gene. In addition, MMP-1 promoter activity was increased as observed with a luciferase promoter construct. By promoter deletion analysis and point mutations they were also able to define AP-1 and ETS binding regions that were vital for the NO induced MMP-1 expression. Further experiments using selective MAPK pathway inhibitors led to the conclusion that this effect was mediated through the ERK and p38 pathways.

**I.10 Hypothesis and Thesis Objectives**

Despite over three decades of research into methods to address the hallmark pathologies that make GBM such a devastating cancer, there still remains a paucity of effective treatment options that can considerably extend the survival of GBM victims. It is becoming more apparent that addressing GBM will require specific targets that are known to influence invasion, tumor related angiogenesis, resistance to apoptosis, and proliferation. It is logical to pursue the MMPs as a potential target because of their well-known abilities to clear ECM, and release bio-active molecules.
Based upon prior work showing that MMP-1 is unique to GBM in the context of the local host environment the central hypothesis underlying the following described studies is that the expression of MMP-1 enhances glioblastoma cell tumorigenicity.

The first portion of this work (Chapter 2) studied the potential interaction between NO and MMP-1. As mentioned above, the enzymes that produce NO, especially the nanomolar producing constitutive isoforms, are enhanced in higher-grade tumors. This is coincident with MMP-1 expression. Work in other systems has presented compelling evidence that NO can indirectly induce the expression of MMP-1 in a pathological setting, yet this has never been demonstrated in the context of GBM. Furthermore, a plethora of pharmacologic inhibitors of NOS are available for use. Therefore in vitro studies were performed using NO donor molecules at a concentration reflective of NO production by cNOS, with the aims of observing enhancements in MMP-1 mRNA and protein expression levels, and induction of GBM cell motility.

Nitric oxide was found to enhance the expression of MMP-1 at both the mRNA and secreted protein levels in GBM cell lines. Motility was also induced in an MMP-1 dependent manner as determined with siRNA-mediated inhibition of MMP-1. Chapter 3 entails further examination into the tumorigenic potential of MMP-1. Glioblastoma cell lines were manipulated to either
stably over-express or knock-down MMP-1 protein production. The central aims of these studies were to assess tumor incidence, growth, and angiogenic potential in vivo. *In vitro* assays were also carried out modeling angiogenesis, as well as proteomic arrays examining possible coincident or downstream players in MMP-1 mediated GBM pathologies.
Chapter 2

Induction of matrix metalloproteinase-1 and glioma cell motility by nitric oxide.

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II.1 Abstract

High-grade gliomas invariably recur due in large part to tumor cells permeating brain in an inaccessible, diffuse manner. Previous work demonstrates that the expression of matrix metalloproteinases (MMP) contributes to this characteristic. Not only can MMPs assist a cell in traversing its environment by clearing extracellular matrix molecules, but they can also impact non-traditional downstream signals that affect a cell’s ability to interact and respond to its surroundings. Contributions to the induction of MMP expression and functional significance in glioma are still under investigation. Evidence in other cancer settings indicates that nitric oxide (NO) may play a role in tumor/cell progression and that NO can influence MMP production. Matrix metalloproteinase-1 (MMP-1), also known as interstitial collagenase, and the constitutive nitric oxide synthases (NOS) have been shown to be over-expressed in high-grade gliomas. The following study investigated the potential involvements of NO with regard to MMP-1 and functional glioma cell movement. With the treatment of a clinically relevant NO donor, sodium nitroprusside (SNP) there was significant induction of MMP-1 mRNA, secreted MMP-1 protein and motility of glioma cell lines within 48 hours. RNA inhibition of MMP-1 through transient transfection of three MMP-1 specific siRNAs revealed a marked abrogation of the NO-mediated induction of
motility. In addition application of the NOS inhibitor \(N_\omega\)-Nitro-L-arginine methyl ester (L-NAME) impaired movement of glioma cells. These data provide evidence for a regulatory axis of high-grade glioma cell movement from NO through MMP-1, with NOS inhibitor results showing promise for future pharmacologic investigation.

**II.2 Introduction**

A hallmark difficulty of high-grade glioma treatment is addressing the diffuse nature of these tumors. Glioma cells invade the normal brain parenchyma intimately making complete surgical resection impossible to achieve. Unfortunately the efficacy of various treatment modalities with high-grade gliomas is most often marginal with primary care focused on palliative management (Louis et al., 2002; Krex et al., 2007; Buckner et al., 2007). Studies have revealed that specific members of a class of extracellular matrix (ECM) degrading enzymes known as the matrix metalloproteinases (MMP) are partially responsible for the increased invasive capacity of high-grade gliomas (Nakano et al., 1995; Fillmore et al., 2001; VanMeter et al., 2001; Stojic et al., 2008). MMPs and their downstream signals are vital components of cell-ECM interaction, growth, communication and survival (McCawley and Matrisian, 2001).
Specifically, it has been demonstrated that MMP-1, also known as interstitial collagenase, is consistently over-expressed at the mRNA and protein levels in high grade astrocytomas while not found in normal brain (McCready et al., 2005).

We posit that the small bio-messenger nitric oxide (NO) may be strongly associated with these changes in MMP-1 expression. Previous findings in other systems indicate that NO can indeed influence the MMP-1 promoter and ultimately expression of the complete protein (Yoshida et al., 2001; Ishii et al., 2002; Choe et al., 2003). Furthermore, there is a discoordinate regulation of the enzymes that endogenously produce NO, the nitric oxide synthases (NOS), in primary CNS tumors, whereby there is an apparent up-regulation of constitutive NOS isozymes and decreases of the inducible NOS (Chapter 1, Table 1.3). Nitric oxide can have paradoxical effects on the cell where on one hand it can confer resistance to apoptosis and enhance proliferation, whereas it can also be toxic through induction of DNA damage responses and inhibition of electron transport (Fukumura et al., 2006; Lam-Himlin et al., 2006; Ridnour et al., 2006). The latter of these issues are likely a result of micromolar, inducible NO production which is most often a host immune response, while the former is attributable to the constitutive NO synthases found in excess in high grade tumors.
In the following study the theory of an axis of glioma invasion and cell movement *in vitro* involving NO and MMP-1 was pursued. Nitric oxide donors, compounds that shed NO in solution, were applied to established malignant glioma cell cultures and subsequent changes in MMP-1 mRNA, protein expression, motility and cell viability were examined. Observed was a marked increase in both MMP-1 message and overall protein in glioma cells treated with NO. There was also significant enhancement of glioma cells’ abilities to traverse and penetrate through a membrane barrier assay at lower NO levels. Viability of these cells was not affected by constitutive-like NO concentrations within the exposure periods used for assessment of cell movement. However, high doses of donors, reflective of inducible NO concentrations swiftly ablated the survival of glioma cells. Cells transfected with siRNA to inhibit MMP-1 protein production resulted in the impairment of NO-induced cell movement. In addition the NOS inhibitor L-NAME educed a striking reduction in glioma motility. These data suggest that there is a regulatory relationship between NO and MMP-1 in malignant glioma cells and that these factors support their highly motile and invasive nature.
II.3 Materials and Methods

Cell Culture

T98G or U87MG cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were monolayer cultured in Dulbecco’s Modified Eagle Medium (DMEM Invitrogen, Carlsbad, CA, USA) with 4.5g/L D-glucose supplemented to 1% penicillin/streptomycin (Invitrogen) and 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA). Cultures were maintained in a 37°C, 5% CO₂ atmosphere. Cells were passed by enzymatic means of a 0.25% trypsin/EDTA solution (Invitrogen), then plated in at least six replicates per condition and allowed to acclimate for 24 hours, upon when NO donor treatment was started. Either sodium nitroprusside (SNP) or S-nitrosoglutathione (GSNO) as donors or Nω-Nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, was applied by dissolution in normal medium or medium without serum. For RNA experiments, U-87MG cells were cultured in T-25 flasks (Nunc, Langenselbold, Germany) containing complete medium. The complete medium was replaced with media containing the different NO related compounds and allowed to incubate for 24h. U-87MG and T98G cells were cultured in 6-well plates (Corning, Lowell, MA, USA) with a density of 200,000 cells per well for the protein analyses, and grown in white-walled 96-well plates (Corning) with an initial density of 2,000 cells per well for viability assays.
RNA Isolation and Analysis

Total RNA was isolated from treated and control cells using TRIzol reagent (Invitrogen) at a ratio of 1mL TRIzol to 10cm² plating surface. An Ultrospec 2000 UV/Visible spectrophotometer (GE Healthcare, Buckinghamshire, UK) set for reading absorbance at 260nm and 280nm was used to determine RNA concentration and quality. Samples were treated with one unit of RQ1 RNase-Free DNase (Promega, Madison, WI, USA) per microgram of RNA at 37°C for thirty minutes. Then a DNase stop solution was added equivalent to 10% of the sample volume and incubated for ten minutes at 65°C. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for MMP-1 and Cyclophilin A were performed in at least five replicates per sample, including a human total RNA reference (Stratagene, La Jolla, CA, USA), at the Nucleic Acids Research Facilities (Virginia Commonwealth University, Richmond, VA, USA) using an ABI 7900 TaqMan Sequence Detector (Applied Biosystems). The mean quantity of Cyclophilin A mRNA was used as a loading reference to compare MMP-1 quantity among samples.

Protein Isolation and Analysis

Conditioned media were aspirated and centrifuged 1000xg for 5 minutes to separate debris. Lysates were obtained by cold extraction with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1%
sodium deoxycholate, 1% NP-40, 0.5% SDS) containing protease inhibitors (EMD Biosciences, Darmstadt, Germany). RIPA buffer was added to each sample well and incubated at 4°C with vigorous agitation for five minutes. Then residual lysates were scraped free from the well and passed thrice through a 22ga needle to shear DNA. The mixture was centrifuged 14000xg for 10 minutes to separate the cytosolic and membrane fractions – the cytosolic fraction was analyzed. Protein concentrations were determined through the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Conditioned media containing 4μg or lysates containing 25 μg of protein were examined by immunoblot. Samples were resolved by SDS-PAGE in 4%-12% Bis-Tris gels (Invitrogen) then transferred to 0.45μm pore nitrocellulose membranes (Invitrogen). Membranes were blocked with tris-buffered saline Tween-20 (TBST), 5% non-fat dry milk for one hour at ambient temperature, then primary probed with either mouse monoclonal α-MMP-1 (MAB901 R & D Systems, Minneapolis, MN, USA) in conditioned media and lysates or rabbit polyclonal α-Cyclophilin A (07-313 Millipore Billerica, MA, USA) for lysates overnight at 4°C. This was followed by TBST rinses (five times four minutes) and secondary application of affinity purified, peroxidase conjugated goat α-mouse (610-1302 for MMP-1) or α-rabbit (611-1302 for Cyclophilin A) IgG antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA) for ninety minutes at ambient temperature. Immunoreactivity was
detected through Enhanced Chemiluminescence Reagents (ECL, GE Healthcare) with subsequent exposure to autoradiographic film (Denville Scientific, Metuchen, NJ, USA). Densitometric analysis of immunoreactivity was carried out using ImageJ (NIH).

**Cell Viability Assays**

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was utilized to assess the number of cells present after NO donor treatment. 2,000 cells were seeded into each well of white-walled 96-well plates (Corning), with six replicates per condition. After one day of allowing the cells to attach and acclimate, complete media were gently aspirated and replaced with media +/- compound and serum. This assay involved incubating complete cultures including media at chosen end points (one to six days) 1:1 volumetrically with a proprietary lysis reagent that produces a luminescent signal directly proportional to the amount of ATP present. A standard curve of known ATP concentrations was analyzed to estimate [ATP] from each culture condition (at least six replicates each). Readings were obtained using a Fluostar Optima plate reader (BMG Labtech, Durham, NC, USA) and the provided software for estimation of ATP concentration.
Membrane Motility Assays

The ChemoTx® System (Neuroprobe, Gaithersburg, MD, USA) was used to gauge changes in movement by the glioma cells. A 96-well microplate format with 8µm pore polycarbonate membranes was utilized. The wells of the provided 96-well microplate were filled with 312.5µL complete medium containing 0-1mM of SNP, GSNO, or L-NAME. Fifty microliters of suspensions of 400,000 cells/mL of NO donor containing media were distributed per test site (membrane area above a well). Membranes were placed over the microplates and cells were allowed to settle and move through the membrane for 24h or 48h. At the desired time point, the membrane was removed and 200µL of media discarded from each well of the plate. The aforementioned ATP viability assay was applied to the well as a measure of cells that moved through and detached below the membrane.

Transient Transfection of MMP-1 siRNA

T98G cells were transfected with Silencer Select pre-designed siRNAs against MMP-1: s8847 sense, GGAAUUCUUUGGCUGAAAtt; s8848, sense CUAGAACUGUGAAGCAUAUtt and s8849 sense, CCAACAAUUUCAGAGAGUAtt (Ambion, Austin, TX, USA). Cells were seeded at a density of 200,000 cells per well in 6-well plates and allowed to settle in complete DMEM for one day. The day subsequent to plating, transfection mixtures were produced for
each condition: normal (no transfection reagent, no siRNA), reagent only, scrambled siRNA, and siRNA, by first aliquotting a volume of 0% FBS Opti-MEM I Reduced Serum Medium (Invitrogen) equivalent to 100µL per replicate. Oligofectamine transfection reagent was added to reach 2% of the transfection mixture volume; tubes were briefly agitated and allowed to incubate for five minutes at ambient temperature. Complexes of siRNA at a 20µM stock concentration were added to the transfection mixtures to reach a final concentration of 15nM, i.e. 1.875µL per 2.5mL of final medium volume in one well of a 6-well plate. The mixtures were briefly agitated and incubated at ambient temperature for twenty minutes. During the incubation, normal media were removed and the cultures were gently rinsed with 0% FBS Opti-MEM I. 500µL Opti-MEM I supplemented to 2% FBS was aliquotted to each well in preparation for transfection. After the twenty-minute incubation 104µL of transfection mixtures were added to respective wells and the plates were incubated in this solution with gentle agitation for four hours at 37°C. At the end of this final incubation the media volumes were brought to 2.5mL with 15% FBS Opti-MEM I. Cells were assayed for protein 72h and membrane invasion 96h post-transfection.

**Statistical Analyses**

JMP 7 software (SAS, Cary, NC) was utilized for the analysis of data. Cell viability, RNA and protein densitometric data were
compared using two-tailed Student’s t-tests for differences among experimental treatment groups and relevant controls. Membrane assay data were tested using one-way ANOVA. If variances were equal, then groups were subsequently compared using Tukey’s honest significant differences test (HSD). Differences between experimental groups and relevant controls were deemed significant if p<0.05.

II.4 Results

A Constitutive Concentration of Nitric Oxide Donor Induces MMP-1 mRNA in U-87MG Cells

This study sought to understand potential influences of NO upon MMP-1 in glioma cell lines. It began by employing a common NO donor Sodium Nitroprusside (SNP). U-87MG cells were cultured under normal conditions (complete medium) until the beginning of SNP treatment. Complete media were removed and replaced with serum free medium with SNP at 100µM. Considering previous literature regarding the preponderance of the constitutive Nitric Oxide Synthases (nNOS and eNOS) in high-grade gliomas, and taking into account that µM NO is indeed cytotoxic, physiologically relevant donor concentrations were applied. Accordingly, 100µM SNP is approximately equivalent to nanomolar ranges of NO (Jaffrey and Snyder, 2001; Ederli et al., 2009).
Total RNA was collected 24h following the media replacement, and subjected to real time PCR analysis by the TaqMan® method at the VCU Nucleic Acids Research Facilities with probes specific for MMP-1 (CCA GGT ATT TCT GGA CTA AGT CCA CAT CTT GC) and cyclophilin A (CACCACATGCTTGCCATCCAACCA) as a reference control. The ratio of MMP-1 to cyclophilin A was significantly greater from cells treated with the NO donor (p<0.001 [Figure 2.1A]). This difference was accounted for by a 14.7-fold increase in MMP-1 mRNA relative to cyclophilin A. To our knowledge this is a novel observation among glioma cell lines, while in agreement with an effect observed recently in human cervical fibroblasts and melanoma cells (Yoshida et al., 2001; Ishii et al., 2002).

**MMP-1 Protein Increases with Sodium Nitroprusside Treatment**

Next were assayed conditioned media from glioma cell cultures treated with SNP to determine if the observations of increased MMP-1 transcript in its [SNP] presence were mirrored at the secreted protein level. Serum free, conditioned media from U-87MG and T98G cells treated with 100µM SNP were collected 24h following initial exposure to SNP. After analysis for protein content (DC Protein Assay, Bio-Rad), conditioned media were equally loaded and resolved by SDS-PAGE under reducing conditions followed by transfer to nitrocellulose membranes and immunoblotting for MMP-1 using a mouse monoclonal anti-human
MMP-1 antibody (R & D Systems). U-87MG cells normally have a low to undetectable level of MMP-1 protein expression and secretion. However, when exposed to 100µM SNP for 24h, U-87MG conditioned media displayed a marked induction of MMP-1 protein compared to untreated controls (Figure 2.1B). Indeed, MMP-1 was scantily detectable in 24h U-87MG controls, with SNP treatment groups having on average 3.3 times more MMP-1 (Figure 2.1C). An increase of MMP-1 was also observed with conditioned media from T98G cells (Figure 2.1B & C) treated with 100µM SNP. Unlike U-87MG, T98G cells express a basal level of MMP-1 protein that is readily detectable in untreated cells’ conditioned media and lysates. Thus we chose to pursue this cell line with subsequent siRNA optimization experiments and functional assays for cell motility.

Increase in Glioma Cell Movement Following NO Donor Treatment

It has been previously reported that MMP-1 is expressed in high-grade gliomas, yet not in normal brain (Nakano et al., 1995; McCready et al., 2005; Stojic et al., 2008). MMPs theoretically facilitate the movement of the cells through the extracellular matrix as well as having other non-traditional targets, e.g. MMP-1 is capable of cleaving IFG-BP thereby releasing a potent growth factor Nakano et al., 1995). Having addressed that NO donor increases MMP-1 message and protein at a constitutive-type
Figure 2.1. NO Induction of MMP-1  

A: MMP-1 mRNA amplified from U-87 MG cells treated with 100µM SNP for 24h; Quantities of MMP-1 were normalized to those of Cyclophilin A amplified in the same samples. Control samples received no SNP treatment.  

B: Representative immunoblots for MMP-1 in the conditioned media of U-87MG and T98G cells treated with 100µM SNP for 24h.  

C: Densitometric analyses of MMP-1 immunoreactivity across four separate experiments.  

*, p<0.001; +, p<0.05.
concentration, we pursued the possibility that the migration of the cells may be a downstream constituent of NO. U-87MG or T98G cells were plated at equal densities onto 8µm pore diameter polycarbonate membranes. Media, both above and below the membranes, contained NO donor and cells were allowed to migrate for 24h to 48h after which the bottom well was measured with an ATP-based viability assay for cells that successfully crossed the barrier. Both U-87MG and T98G cells in the presence of low dose SNP (100µM) more successfully relocated to the lower chamber within 48h. U-87MG cells only displayed a modest, though significant, increase of 21.6% (Figure 2.2). On the other hand, T98G cells revealed a 400% or greater induction of movement with 100µM SNP (Figure 2.3A & B). We performed the same assessment using a physiological NO donor, GSNO, at donor concentrations equivalent to those used for SNP. 100µM GSNO revealed a significant increase of cells across the membrane of 35.6% (p<0.001, Figure 2.4A) in T98G cells alone. A high dose of either donor (1mM) revealed a knock down of at least 80% in both U-87MG and T98G relevant to this assay (p<0.001, U-87MG: Figure 2.2; T98G: Figure 2.4).

The reaction of these cells in this assay in response to L-NAME was also tested. Treatment with the NOS inhibitor, regardless of concentration, led to migratory impediment greater than 40% compared to control (p<0.001, [Fig. 2-3C]) with the
Figure 2.2. Membrane motility assay of U-87MG cells exposed to varying concentrations of the NO donor SNP. There was a modest but significant increase in motility with 100µM SNP, which reflects constitutive-type NOS production. The apparent motility of U-87MG cells in the presence of 1mM SNP was substantially reduced. *p<0.001
Figure 2.3. Membrane motility assays of T98G glioma cells in the presence of SNP and a NOS inhibitor, L-NAME. **A & B:** T98G cells subjected to 100µM SNP for 24h (**A**) or 48h (**B**) displayed an induction of cell motility versus untreated controls. **C:** T98G cells with the arginine analog NOS inhibitor L-NAME at all doses showed significantly decreased motility with the maximal effect at 1mM. *, p<0.001.
Figure 2.4. T98G membrane motility assays with high dose NO donor.  
A. Membrane motility assays of T98G cells exposed to varying concentrations of the physiological NO donor GSNO. Motility was significantly increased with 100µM GSNO and reduced with 1mM.  
B. Membrane motility assays of T98G cells demonstrating the apparent significant decrease in motility with 1mM SNP. *p<0.001
maximal impairment of 53.1% at 1mM. These data implicate the endogenous production of NO as an important factor contributing to glioma cell movement.

To address the hypothesis that the previous observations could be due to changes in viability/proliferation with NO exposure rather than cell movement, the same ATP based viability assays were performed over 5 days in the presence of NO donors in normal culture conditions. Lower concentrations (1µM and 100µM) of NO donor (either SNP or GSNO) did not elicit significant changes in glioma cell viability compared to untreated controls. However, with 1mM SNP there was cell death within hours of initial exposure and by the first day culture viability of glioma cells fell approximately 80% (p<0.001 [Figure 2.5A & Figure 2.6]) compared to control, followed by undetectable levels of ATP by the fifth day.

Finally, having noted a marked decrease in motility with the NOS inhibitor L-NAME, glioma cell viability in its presence was assessed to exclude compound related mortality as the cause for those observations. There were no significant changes in viability among treatment groups across times of exposure when motility assays were also performed, but by the fifth day there were modest yet significant reductions in viability with ≥100µM (Figure 2.5B).
Figure 2.5. ATP viability assays of T98G and U-87MG cells  

A. Viability in the presence of NO donor (either SNP or GSNO) over time at varying concentrations. Constitutive-like donor concentrations ≤100µM did not significantly affect viability, however 1mM donor inclusion reduced culture viability substantially (p<0.001).  

B. Viability in the presence of the NOS inhibitor L-NAME over time at varying concentrations. By Day 5, there were modest yet significant reductions in cultures treated with ≥100µM L-NAME (p<0.001).
A  Glioma Cell Viability in the Presence of NO Donor

B  Glioma Cell Viability in the Presence of L-NAME
Figure 2.6. Phase contrast microscopy of U-87MG cells over five days in the presence of SNP at varying concentrations (100X magnification). The X-axis represents time points in culture with SNP increasing from left to right, and the Y-axis displays different concentrations of SNP increasing from top to bottom. It can be seen within hours (bottom left corner) that treatment with the 1mM dose of NO donor is cytotoxic, while all other treatments appear grossly similar to the control (no treatment – top row).
Transient Inhibition of MMP-1 Protein in Glioma Cells via siRNA Blunts the NO Induction of Glioma Cell Movement

Next it was sought to inhibit MMP-1 to determine if it had an impact on the apparent NO stimulated migration, as both MMP-1 mRNA and protein are concomitantly increased with NO donor presence. First, several different pre-designed siRNAs for MMP-1 were tested: s8847, s8848 and s8849 (Silencer Select, Ambion, see II.3 Methods for sequences). T98G cells were chosen as an initial target since regardless of NO input or serum condition they produce a readily detectable basal level of MMP-1 protein. Oligofectamine transfection of all siRNAs to a final concentration of 15nM reduced MMP-1 immunodetection at least 50% (Figure 2.7). The s8849 siRNA provided the strongest knock down at over 95% compared to normal (untransfected) T98G control (Figure 2.7A & C). The three siRNAs were also pooled, each contributing equally to the final RNA concentration in culture, and with combination a similar MMP-1 protein inhibition was observed (Figure 2.7B). Transfected T98G cells were subjected to the aforementioned membrane assay in the presence or absence of 100µM SNP for 48h, corresponding to 96h post-transfection. The inhibition of MMP-1 protein production did not prohibit basal movement. Indeed, regardless of siRNA condition it was observed that the transfection procedure affects the glioma cells by mildly inducing movement.
Figure 2.7. Transient transfection of MMP-1 siRNA into T98G cells at a final concentration of 15nM. A: Representative blot of T98G lysates from: N, Normal (untransfected control); B, transfection buffer (Oligofectamine); Scr, scrambled; or three different siRNAs specific for MMP-1, s8847, s8848, s8849. The immunoreactive banding at 52kDa corresponds to MMP-1 while the detection at 17kDa is the loading control, cyclophilin A. B: Representative blot of T98G lysates where the three siRNAs were equally pooled. C: Densitometric ratios of MMP-1:Cyclophilin A immunoreactivity from the blot in A quantifying siRNA mediated knockdown of MMP-1.
All groups given SNP were significantly more motile than normal T98G. However, when the differences among each group and relevant controls were calculated, representing the extent of NO-mediated induction, it was observed that the NO induction of movement was abrogated at least 60% in cells that received siMMP-1 (p<0.001, [Figure 2.8]), and that this effect was significantly less than either transfection control (p<0.05).

II.5 Discussion

The present study demonstrates a role for nitric oxide in the motility of high-grade, malignant gliomas. A glioma cell’s capacity for movement is essential to the insidious, invasive nature of these tumors. Herein it was shown that with a NO donor concentration reflective of NO production by the constitutive nitric oxide synthases there is a clear induction of both MMP-1 mRNA and secreted protein from glioma cells in vitro. These observations alone are important to consider because of previous evidence presenting that high-grade gliomas over-express NOS and MMP-1, particularly since MMP-1 is not found in normal brain parenchyma (McCready et al., 2005; Introduction Table 3). These experiments delineate a potentially strong link between both enzymes, as they appear dis coordinately regulated between high-grade, malignant gliomas
Figure 2.8. Abrogation of NO-mediated induction of glioma cell movement. When examining the differences between +/− SNP in each group, siMMP-1 transfection reduces the SNP movement induction by 60.33%. The transfection procedure alone has an effect as both controls also diminished induction; however, siMMP-1 is significantly less than either of them. *, p<0.001 compared to Normal; +, p<0.05 compared to either control.
and normal brain. Similar observations in human melanoma cells and cervical and dermal fibroblasts have been reported (Yoshida et al., 2001; Ishii et al., 2002; Choe et al., 2003).

Here it is revealed that treatment of glioma cultures with NO donors reproducibly contributes to the in vitro invasive phenotype of glioma cells. This was only observed for donor compound concentrations that presumably bear an amount of NO typical of the cNOS production range — less than one micromolar NO. When reaching a donor concentration reflective of iNOS production — greater than micromolar range — there was a strong decrease in cell viability.

While NO clearly induced MMP-1 in glioma cells as well as elevating motile in vitro response it was still necessary to implicate MMP-1 as a potential intermediary. Thus the inhibition of MMP-1 protein production was pursued through the transient transfection of three siRNAs (Silencer Select Pre-Designed, Ambion) targeting exons three, five and nine of the MMP-1 transcript. All siMMP-1 effectively knocked down MMP-1 protein detection 55% to 95% at a 15nM final RNA concentration. Transiently transfected glioma cells subjected to the membrane assay showed no reduction in apparent movement without SNP across transfection groups. Sodium nitroprusside educed the same responses as previously observed in glioma cells, but also enhanced the movement of siMMP-1 transfected cells versus
control. However, upon examining the differences between each group +/- SNP, it was revealed that the NO-related induction was severely impaired in the siMMP-1 group. While this was also seen among transfection controls, the siMMP-1 cells were still significantly less stimulated to translocate through the membrane. Though the stress of transfection alone has an impact, these data lead us to conclude that MMP-1 is likely part of the NO-stimulated motile response.

Aside from their roles as extracellular matrix clearers, MMPs have a multitude of non-traditional substrates. Among these with regard to MMP-1 is the ability to cleave Perlecan, IGFBP-2/3, and MMP-1/2 to release FGF, IGF and active MMP-1/2 respectively (McCawley and Matrisian, 2001). Furthermore, recent evidence points to MMP-1 as assisting in resistance to lamin A degradation during apoptosis (Limb et al., 2005). Therefore, it was necessary to test the viability of these cells in the presence of NO-stimulated MMP-1. Our ATP-based assays showed no major differences in cell viability with the constitutive-like NO donor concentrations. One millimolar NO donor conferred swift death within 24 hours of beginning the assays. This suggests that toxicity conferred by micromolar NO contributes to the lack of movement at those doses. There were no changes in viability observed within two days of exposure to L-NAME regardless of concentration.
Taken together, the data presented in this study define aspects of NO and MMP-1 as intriguing players in high-grade malignant gliomas. This underscores the importance of MMPs in central nervous system neoplasia. In a recent report, Hodgson and colleagues (2009) show an amplification of gene transcription for MMP-1 and MMP-13, another collagenase, in a subset of GBM clinical samples. Additionally MMP-2 can be an important responder to NO exposure of U-87MG cells (Lin et al., 2008). The same study by Lin and coworkers also presents the inhibition of invasion through treatment of dexamethasone, a known inhibitor of iNOS, thereby providing some evidence that iNOS may indeed have a larger role in high-grade glioma invasion.

It is imperative to examine these characteristics because of the dichotomous roles of NO pathophysiology. The effects of NO on cancer cells have emerged as especially setting/tissue dependent (Fukumura et al., 2006; Ridnour et al., 2006). Indeed, recently it has been demonstrated that a concentration of SNP used in this study (100µM) inhibited invasion of human prostate and bladder carcinoma cells (Wang et al., 2007). The effects seen there were shown to have mitochondrial dependence, bearing importance upon the examination of cancer cell viability as a corollary to movement and invasion. Interestingly it was also reported that HIF-1α was an effector of hypoxia-mediated
invasion, yet it was ablated by the presence of NO. However, tumor associated hypoxia is a key feature of high-grade glioma progression and in some species it is known that HIF-1α activates the transcription of iNOS. Nitric oxide production is furthermore intertwined with VEGF over-expression common to high-grade astrocytomas and is a known associate of endothelial cell proliferation (Kaur et al., 2005). These discordant concepts shed light on the importance of understanding the differences NO can confer on distinct cancer cell types.

Finally, previous investigations by Gu and colleagues (2001) have shown the permanent activation of the latent pro-MMP-9 via S-nitrosylation. This form of post-translational modification, which targets susceptible cysteine thiols, can also inhibit enzyme activity, for instance the inhibition of certain caspases (Fukumura et al., 2006). However, MMPs are manufactured in a latent pro-form, which is dependent on the disposition of the pro-domain from a coordinate thiol for enzyme activity — the cysteine switch. Unfortunately the reliable detection of S-nitrosothiols is still unachievable. Our attempts using the method of Jaffrey and Snyder (2001) have proven fruitless with regard to MMP-1. Regardless, while this avenue might provide unique insights into MMP-1 activation, the concentration of NO required to do so is likely cytotoxic and beyond that which GBM cells would produce.
Nitric oxide synthase inhibitor use is a promising prospect, as seen with a reduction in glioma cell motility in this work. However unlike MMP-1, cNOS isoforms are not locally unique to GBM. The systemic use of NOS inhibitors is known to cause hypertension by inhibiting endothelial cell dependent vasorelaxation. The development of specific NOS inhibitors and targeting techniques will be a prudent matter in the pursuit of this possible therapy. As such the subsequent portion of this work (Chapter 3) focuses on the direct effects of MMP-1 modulation on GBM cell tumorigenicity, and another hallmark pathology: tumor-induced angiogenesis.
Chapter 3

Matrix metalloproteinase-1 enhances glioblastoma tumorigenicity, tumor-related angiogenesis and is inversely associated with tissue inhibitor of metalloproteinases-4

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III.1 Abstract

Matrix metalloproteinase-1 (MMP-1) is a zinc dependent endopeptidase over-expressed in human glioblastoma multiforme (GBM) and has been shown to influence glioma cell migration. Herein the study of this enzyme with respect to GBM cell tumorigenicity and tumor related angiogenesis is continued. Glioblastoma cell lines were stably altered to either over-express or knock down MMP-1 protein production. Subcutaneous inoculation of athymic nude mice with these cells revealed that the presence of MMP-1 significantly increased tumor incidence and size. Three-dimensional human umbilical vein endothelial cells (HUVECs) co-cultured with glioblastoma cells were used to model angiogenesis in vitro. Endothelial projection formation and organized growth were significantly increased with both MMP-1 expression and exogenous application of recombinant protein. Immunohistochemical analysis of tumors for CD31, specific for endothelial cells, elucidated a substantial recruitment of endothelium in MMP-1 expressing tumors. Further in vitro angiogenesis analysis with targeted antibody arrays indicated an inverse expression of certain anti-angiogenic factors with respect to MMP-1 expression. The most notable of these changes was a significant increase in tissue inhibitor of metalloproteinases (TIMP) -4 expression in the absence of MMP-1, as validated by immunoblot. Angiogenesis assays using TIMP-4
confirm its anti-angiogenic properties. Taken together these
data indicate that MMP-1 contributes to GBM cell tumorigenicity,
possibly via the regulation of anti-angiogenic molecules. In
addition to its pro-invasive property, this pathologically local
enzyme is also pro-angiogenic. We propose that these key
features highlight MMP-1 as a promising target for in depth pre-
clinical study and targeting in GBM.

III.2 Introduction

The most aggressive type of malignant primary brain tumor,
glioblastoma multiforme (GBM), is also the most common,
accounting for about half of all malignant gliomas. Even with
current standard therapies — surgical de-bulking, irradiation,
temozolomide — patient survival beyond two years post-diagnosis
remains low and symptoms grim (Louis et al., 2002; Buckner et
al., 2007; Fisher et al., 2007). While the paradigm of
temozolomide treatment concomitant with radiotherapy and six
months adjuvant thereafter has resulted in definitive extension
of survival time (Stupp et al., 2005; Jeon et al., 2009), more
recent strides are being taken in advancing treatment based upon
targeting specific angiogenic pathways and related signaling
cascades (Gillespie et al., 2007; Mathieu et al., 2008; Gu et
al., 2009; Kärrlander et al., 2009). Indeed, profuse
endothelial hyperplasia and invasion are defining
characteristics of GBM. Intrinsic to these pathological
features are extracellular matrix (ECM) cues and activities. In particular, the expression of matrix metalloproteinases (MMPs) is vital to the mediation of ECM composition, tumor cell navigation, and the release of bioactive molecules that can be pro-tumor (McCawley and Matrisian, 2001).

It has been previously demonstrated that the expression of MMP-1, also known as interstitial collagenase, is an important characteristic of GBM. Not normally found in the developed CNS, MMP-1 is profoundly over-expressed in GBM patient samples at the mRNA and mature protein levels (McCready et al., 2005; Stojic et al., 2008; Hodgson et al., 2009). Recently it has been shown that MMP-1 can induce angiogenesis (Blackburn and Brinckerhoff, 2008). Furthermore, this enzyme has been found to enhance motility and invasion in GBM cells and other cancer settings (Benbow et al., 1999; Yoon et al., 2003; Blackburn et al., 2007; Blackburn et al., 2009). The control of MMP-1 expression is multifaceted, for instance it is theorized that promoter activation can result from the prevalence of a single nucleotide polymorphism that introduces an element responsive to the MAPK cascades (Rutter et al., 1998; McCready et al., 2005). Nitric oxide induces the expression of MMP-1 and glioma cell motility (Pullen and Fillmore, 2010 [Chapter 2]), and can post-translationally activate at least one other MMP (MMP-9) via S-nitrosylation (Gu et al., 2002; Harris et al., 2008).
Additionally, all MMPs can be controlled by their endogenous inhibitors: the tissue inhibitors of metalloproteinases (TIMPs), a family of four proteins that bind MMP active sites, thereby preventing proteolytic cleavage of substrates. Contrary to its namesake, TIMP-2 has been shown to interact with MT-1 MMP to facilitate the activation of MMP-2 (Hernandez-Barrantes et al., 2000; Wang et al., 2000), which is an indicator of poor prognoses in multiple cancers (Björklund and Koivunen, 2005). Comparably less is known about the TIMPs and their potential functions in GBM. It has been reported that differential expression levels of TIMP-1 and TIMP-4 within high-grade malignant gliomas might correlate with longer survival (Groft et al., 2001; Aaberg-Jessen et al., 2009). Furthermore, TIMPs are regarded as inhibitors of angiogenic processes (Brew et al., 2000; Rege et al., 2005).

Despite these observations, there have been few functional analyses into the importance of MMP-1 in GBM. While the examination of MMPs might have been abandoned by many due to the unacceptable side effects of an inhibitor in clinical trial in the past decade (Groves et al., 2002), we submit that the continued investigation of these enzymes will contribute to the collective understanding of GBM.

The experiments described herein were performed with a focus on the functional impacts of MMP-1 expression in GBM
cells. Expression of MMP-1 not only enhanced the formation of tumors, but also their sizes. Glioma cells and recombinant MMP-1 clearly induced endothelial cell morphology indicative of angiogenesis in an in vitro model (Chen et al., 2009), in a MMP-1 dependent manner. Subsequent immunohistochemical examination of model tumor specimens elucidated a striking recruitment of endothelium throughout MMP-1 over-expressing tumors. Interestingly, proteomic analyses using antibody arrays against angiogenesis related factors revealed anti-angiogenic expression patterns inverse to the levels of MMP-1. By far the greatest of these changes was in the detection of TIMP-4, which significantly increased with the removal of MMP-1. The anti-angiogenic role of TIMP-4 was confirmed with the in vitro angiogenesis model. These data serve as evidence in support of MMP-1 and related factors as potential, focused targets to GBM angiogenesis and invasion, and warrant continued investigation into their actions and controls in the context of GBM biology.

III.3 Materials & Methods

Cell Culture
U251 MG and T98G cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured as previously described (Pullen and Fillmore, 2010 [Chapter 2]). Human umbilical vein endothelial cells (HUVEC) and their growth
medium (EGM-2) were obtained from Cambrex Bio Science (Walkersville, MD) and grown as in Chen et al., 2009.

**Over-expression and knock-down of MMP-1 protein**

U251 MG cells were stably transfected with the pIREs-GFP-MMP-1 vector containing sequences for green fluorescent protein and MMP-1, as well as G418 resistance. Two million cells were transfected with 1µg DNA using the Oligofectamine™ transfection method according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Three days after transfection, medium containing 500µg/ml G418 (Invitrogen) was added to the U251 MG-vector control and MMP-1 over-expressing transfectants. The G418-resistant cells were selected and grown for further experiments. Afterwards, these cells were stably maintained in complete DMEM supplemented with 400µg/ml G418.

T98G cells were stably transduced with MISSION™ lentiviral shMMP-1 particles (Sigma-Aldrich, St. Louis, MO) to knock down production of the protein. Transduction was carried out according to the manufacturer’s protocol using a multiplicity of infection equal to fifteen. Stable colonies were selected over a period of one month under pressure of 1µg/mL puromycin (Sigma-Aldrich) supplemented complete DMEM. After confirmatory immunoblots of MMP-1 knock-down (>50% by semi-quantitative
densitometry), stable clones were maintained under the same puromycin selection pressure.

**Immunoblot**

Cell culture proteins were isolated with RIPA buffer as described previously (Pullen and Fillmore, 2010 [Chapter 2]). Tumor lysates were obtained by first grinding liquid nitrogen snap-frozen specimens to a fine powder, and then by applying tissue protein extraction reagent (T-PER, Thermo Scientific, Rockford, IL) according to the manufacturer’s protocol. Total protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA).

Samples were resolved by SDS-PAGE as in Pullen and Fillmore, 2010 (Chapter 2). Primary antibodies and respective dilutions were: 2µg/mL mouse α-human MMP-1 (MAB 901, R & D Systems, Minneapolis, MN), 2µg/mL mouse α-human TIMP-4 (MAB974, R & D Systems), or 1:5000 rabbit α-human Cyclophilin A (07-313, Millipore, Billerica, MA). A dilution of 1:3000 HRP-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA) was utilized; either goat α-mouse (610-1302) or α-rabbit (611-1302) IgG. Immunoreactivity was detected with Enhanced Chemiluminescence Reagents (ECL, GE Healthcare, Buckinghamshire, UK) followed by exposure to and development of autoradiographic film (Genesee Scientific, San Diego, CA).
Films were digitally scanned and densitometric analyses were performed using ImageJ (NIH, Bethesda, MD).

**In vivo Tumor Growth**

The growth of U251 MG and T98G cells was assessed in athymic nude mice (Hsd: Athymic Nude-Foxn1nu, Harlan Sprague-Dawley, Indianapolis, IN) using a subcutaneous flank inoculation model. Cells were rinsed three times with 1X DPBS (Invitrogen) then suspended at $2.5 \times 10^6$ cells/mL (U251 MG) or $5 \times 10^6$ cells/mL (T98G) in a 1:1 plain DMEM (no supplements) to growth factor reduced Matrigel™ (BD, Franklin Lakes, NJ) solution. Mice were inoculated subcutaneously in their hind flanks with 200 µL of this suspension in three separate cohorts per cell type (U251 MG controls, U251 MG over-expressor, T98G controls, T98G knock-down); to attain eight to ten overall inoculates per cell type. Tumor take was assessed by the formation of a palpable mass within seven days post-inoculation, and growth was assessed over three weeks using external caliper measurements according to the formula: $(L \times W^2) \div 2$, where $L$=longest axis and $W$=shortest axis. Representative tumors were excised and either fixed in 10% formalin for downstream histological analyses, or snap-frozen in liquid nitrogen and stored -80°C for subsequent immunoblots. The VCU Institutional Animal Care and Use Committee approved these experiments in accordance with federal and local guidelines under protocol number AD20136.
**In vitro Angiogenesis Model Assay**

Our laboratories have developed an *in vitro* model of glioma-induced angiogenesis, the method of which is explicitly detailed in Chen *et al.*, 2009. The principals of the assay involve first coating Cytodex™ 3 microcarrier beads (Amersham-GE Healthcare) with HUVECs, then immobilizing the coated beads in a three-dimensional fibrin gel environment. The gels were overlaid with complete EGM-2, which can include desired soluble factors of study, or other cells for co-culture. Previously, it has been demonstrated that HUVEC sprout formation and growth from the beads can be induced with VEGF or glioma cell co-cultures. Without angiogenic cues the HUVECs randomly migrate away from the beads over time. This assay was performed for this work with co-culture of the aforementioned stably altered glioma cells. Furthermore, beads were also cultured in the presence of 10ng/L recombinant human (rh)MMP-1 (901-MP-010, R & D Systems), and 50ng/L rhTIMP-4 (974-TSF-010, R&D Systems). After five days, with refreshment of media at the end of day two, the number of sprouts per bead was counted and lengths measured using SPOT Advanced v3.2.4 software and camera (Diagnostic Instruments, Inc.) calibrated to the objective magnification settings of an Olympus CK40 microscope.
Histologic and Immunohistochemical Processing

Representative in vivo tumors were taken and post-fixed in 10% formalin. Specimens were paraffin embedded and 6µm thick serial sections used for H&E staining and CD31 (PECAM-1) immunohistochemistry. Antigen retrieval was conducted by heating sections to 100°C for 20 minutes. Rat anti-mouse CD31 primary antibody (550274, BD Pharmingen) was applied to the samples in a 1:50 dilution in horse serum, followed by incubation with a biotinylated rabbit α-rat secondary (BA-4001, Vector Laboratories, Burlingame, CA), and development by the DAB method with the VECTASTAIN® Elite ABC kit according to the manufacturer’s protocol (Vector Laboratories). Sections were counterstained with hematoxylin.

Positivity for CD31 was determined using an automated Ariol® slide quantification platform (Genetix Corp., San Jose, CA). Briefly, the computer operated Olympus BX61 microscope loaded individual slides and scanned tissue sections at 20X objective magnification. Once all scanning was completed, quantification of CD31 positivity was calculated based upon multiple color channels as well as the morphology of immunoreactivity. These values were reported in square microns, and then transformed to a percentage of total area analyzed per region of interest.
Proteomic Profiler Antibody Arrays

Human angiogenesis antibody arrays (ARY007 R&D Systems) were utilized according to the manufacturer’s protocol. These arrays consisted of nitrocellulose membranes spotted in duplicate with antibodies raised against 55 angiogenesis related proteins (Table 3.1). Briefly, RIPA lysates from multiple passages of the glioma cell lines within each condition were pooled. Each condition, e.g. T98G control or T98G MMP-1 knock-down, was incubated with a biotinylated detection antibody, while membranes were blocked, at ambient temperature prior to overnight incubation at 4°C with individual membranes per pooled condition. Membranes were washed and incubated with streptavidin-HRP, followed by another wash and immersion in ECL Reagents. Immunoreactivity was detected by membrane exposure to autoradiographic film. Developed films were densitometrically analyzed in the same fashion as immunoblots.

Statistics

JMPv8 software (Cary, NC) was utilized for the statistical analyses of data. Student’s t-test was performed for comparisons among groups in experiments reported with continuous data. For categorical tumor take data, Fisher’s Exact Test was calculated. Differences were deemed significant for p<0.05.
Table 3.1. Angiogenesis proteome profiler. Antibodies directed against the following angiogenesis related proteins were utilized in the proteomic profiler arrays.
<table>
<thead>
<tr>
<th>Activin A</th>
<th>FGF-7/KGF</th>
<th>PD-ECGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMTS-1</td>
<td>GDNF</td>
<td>PDGF-AA</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>GM-CSF</td>
<td>PDGF-AB/PDGFB-BB</td>
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<tr>
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<td>HB-EGF</td>
<td>Persephin</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td>HGF</td>
<td>CXCL4/PF4</td>
</tr>
<tr>
<td>Angiostatin/Plasminogen</td>
<td>IGFBP-1</td>
<td>PIGF</td>
</tr>
<tr>
<td>Amphiregulin</td>
<td>IGFBP-2</td>
<td>Prolactin</td>
</tr>
<tr>
<td>Artemin</td>
<td>IGFBP-3</td>
<td>Serpin B5/Maspin</td>
</tr>
<tr>
<td>Tissue Factor/Factor III</td>
<td>IL-1 beta</td>
<td>Serpin E1/PAI-1</td>
</tr>
<tr>
<td>CXCL16</td>
<td>CXCL8/IL-8</td>
<td>Serpin F1/PEDF</td>
</tr>
<tr>
<td>DPPIV/CD26</td>
<td>LAP (TGF-beta 1)</td>
<td>TIMP-1</td>
</tr>
<tr>
<td>EGF</td>
<td>Leptin</td>
<td>TIMP-4</td>
</tr>
<tr>
<td>EG-VEGF</td>
<td>CCL2/MCP-1</td>
<td>Thrombospondin-1</td>
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<tr>
<td>Endoglin/CD105</td>
<td>CCL3/MIP-1alpha</td>
<td>Thrombospondin-2</td>
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<tr>
<td>Endostatin/Collagen XVIII</td>
<td>MMP-8</td>
<td>uPA</td>
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<td>MMP-9</td>
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</tr>
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<td>Pentraxin 3</td>
<td>VEGF-C</td>
</tr>
<tr>
<td>FGF-4</td>
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</table>
III.4 Results

Stable manipulation of MMP-1 expression and glioma cell tumorigenicity

Previously our laboratories reported the over-expression of MMP-1 in GBM patient samples (McCready et al., 2005) as well as glioma cell dependence on MMP-1 expression for NO-induced motility (Pullen and Fillmore, 2010 [Chapter 2]). Matrix metalloproteinases are not simply regarded as ECM clearance enzymes, but are also potent liberators of bioactive, pro-tumor factors. Prior experiments examined the control of MMP-1 expression in a transient, in vitro context, thus the next evolution of this work was to establish stable over-expression and knock-down of MMP-1 protein in different glioblastoma cell lines, and then to assess the impact of MMP-1 on glioblastoma cell tumorigenicity in vivo.

U251 MG cells do not produce an appreciable level of MMP-1 under standard culture conditions. Therefore this cell line was chosen for the stable over-expression of MMP-1. This was successfully accomplished using a pIREs-GFP vector with stable maintenance under the selection pressure of G418 (Figure 3.1A). Conversely, T98G cells produce MMP-1 under standard conditions. These cells were transduced with various lentiviral clones containing MMP-1 shRNA sequences and puromycin resistance.
Clone 36 (TRCN0000003336, NM_002421.x-1702s1c1) proffered complete knock-down of MMP-1 protein as assessed by immunoblot (Figure 3.1B).

These MMP-1 manipulated cells were then examined in vivo. Subcutaneous flank inoculation of athymic nude mice was chosen, because it models a non-permissive growth environment in which tumor cells must appropriately sense and respond to their surroundings to maintain viability, and provides an efficient mode of direct tumor growth observation. The first grossly observable effect of changes in MMP-1 expression was an impact on the ability of glioma cell inoculates to form tumors. All T98G control inoculates produced palpable tumors; however, fewer than 40% of those with stable MMP-1 knock-down formed (p<0.05, Table 3.2). Similarly, with regard to MMP-1 expression, U251 MG control inoculates had approximately 50% tumor take, whereas 100% of MMP-1 over-expresser inoculates developed tumors (p<0.05). Furthermore, the tumors that did form in the T98G MMP-1 knock-downs and U251 MG controls had significantly reduced volumes compared to their respective MMP-1 expressing counterparts (p<0.05, Figure 3.2A&B). Eventually all T98G MMP-1 knock-down tumors regressed. To determine if MMP-1 expression was maintained, representative tumors were excised, snap frozen, and proteins isolated for MMP-1 immunoblot. Greater than 50% MMP-1 knock-down was observable in T98G tumor lysates, while
Figure 3.1. MMP-1 immunoblots of stable MMP-1 manipulation (cyclophilin A used as a loading control) demonstrating A, U251 MG cells that were stably transfected with a pIRES-GFP-MMP-1 G418 resistant cDNA vector to over-express MMP-1 protein. B, T98G cells that were stably transduced with lentiviral particles coding for shMMP-1 and puromycin resistance for MMP-1 protein knock-down. P: parental; VC: Vector Control; MMP-1+: MMP-1 over-expressor; NT: non-target shRNA lentivirus; sh: MMP-1 knock-down lentivirus.
Table 3.2. Categorical palpable tumor formation within seven days post-inoculation. $T_p<0.05$
<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Tumor Take by 1 week(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U251 Control</td>
<td>55.6 (n=9)</td>
</tr>
<tr>
<td>U251 + MMP-1†</td>
<td>100 (n=9)</td>
</tr>
<tr>
<td>T98G Control</td>
<td>100 (n=10)</td>
</tr>
<tr>
<td>T98G shMMP-1†</td>
<td>37.5 (n=8)</td>
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</tbody>
</table>
there was greater than twice the level of immunodetection in U251 MG MMP-1 over-expressers compared to relevant controls (Figure 3.2C&D). It should be noted that MMP-1 production can be induced in U251 MG control cells in an in vivo context. These data suggest that MMP-1 expression is vital for glioma cell tumorigenicity.

Influence of MMP-1 expression in an in vitro model of angiogenesis

One of the defining characteristics of GBM is extensive tumor-associated endothelial hyperplasia. Advances in targeting factors associated with angiogenic cascades in GBM have begun to display clinically meaningful treatments for this disease. Recently, it has been proposed that MMP-1 could also fulfill a pro-angiogenic role in cancer (Blackburn et al., 2007 & 2009). Therefore we pursued the examination of glioma cell associated angiogenic induction with respect to MMP-1 expression in an in vitro model established in our laboratories (Chen et al., 2009). Cytodex™3 microcarrier beads were coated with HUVECs then immobilized in a three-dimensional cell culture environment. HUVECs randomly migrate and fall away from the microcarrier beads when only basic growth medium is placed over the gels. With the co-culture of MMP-1 expressing glioma cells there was an induction of HUVEC projection formation and growth from the coated beads. Specifically, over-expression of MMP-1 in U251 MG
**Figure 3.2.** *In vivo* experiments on the tumorigenicity of glioblastoma cells with altered MMP-1 expression. **A,** growth of T98G inoculates over three weeks. **B,** growth of U251 MG inoculates over three weeks. Growth is presented as mean volume (mm³) by external caliper measurement according to the modified ellipsoid formula \((L \times W^2)/2;\) \(n=8-10\) per condition, divided among three separate cohorts \(*p<0.05.\) **C** (T98G) and **D** (U251 MG) are MMP-1 immunoblots on representative tumor lysates twelve days post-inoculation, demonstrating the continued, stable alteration of MMP-1 expression in vivo.
induced greater sprout length versus controls (p<0.05, Figure 3.3A) and knock down of MMP-1 expression in T98G reduced length (p<0.05, Figure 3.3B). When rhMMP-1 was included with the growth medium, without glioma cell co-culture, there was an increase in HUVEC projection number and length (p<0.05 Figure 3.3C&D), indicating that in vitro MMP-1 is a glioma cell related, pro-angiogenic component.

**In vivo assessment of glioma related MMP-1 pro-angiogenic potential**

Based upon the in vitro model results we hypothesized that MMP-1 expression would contribute to an angiogenic response from glioblastoma cells in vivo. Representative tumors from the initial tumorigenicity experiments were excised and post fixed in 10% formalin for the purpose of histological examination. T98G MMP-1 knock-down did not produce appreciable tumor quantities, both in number and size, for multiple reliable analyses. Hematoxylin & eosin simple stained paraffin embedded sections suggested qualitatively that there were more vascular structures present in U251 MG MMP-1 over-expressing tumors (Figure 3.4A&B), especially in peripheral tumor associated stroma. Thus U251 MG control and MMP-1 over-expressing tumors were subjected to immunohistochemistry for a more definitive observation of tumor-associated endothelium and potential quantification.
Figure 3.3. *In vitro* angiogenesis assays in the context of MMP-1 expression. **A** (U251 MG) and **B** (T98G) present endothelial cell projection length from endothelial coated Cytodex™ beads co-cultured in a three-dimensional environment with glioblastoma cells. **C**, average number of projections per bead in the presence or absence of rhMMP-1 (10ng/L) and **D**, length of those projections, there was no co-culture involved in C&D. *p<0.05.*
Also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), CD31 is acknowledged as a marker of endothelium, the immunoreactivity of which is often utilized for the analysis of microvascular density in pathological specimens. A monoclonal antibody raised against mouse CD31 was used to detect the endogenous recruitment of vessels in vivo. Immunoreactivity was specific for vascular endothelium and not other ductile tissue within the sections. Furthermore, there was a profound increase in CD31 immunoreactivity within MMP-1 over-expressing tumors compared to U251 controls (Figure 3.4C&D). Vast, anastamosing networks of positivity were observed in MMP-1 over-expressers that were not seen in low MMP-1 expressers. Indeed, these networks were so profuse that quantification of discrete blood vessels became difficult. Therefore the use of an automated Ariol® slide quantification platform was pursued. The instrument software was trained to recognize true positive immunoreactivity through the utilization of multiple color channels, and importantly through the morphology of immunoreactivity. After scanning and analyzing immunohistochemical sections including negative (no primary antibody) controls, it was determined that on average 3.26% of any given region of interest in a U251 MG control tumor was CD31/vessel-shape positive. The MMP-1 over-expressing tumors
Figure 3.4. Analysis of endogenous vascular recruitment in MMP-1 over-expressing tumors. A (U251 MG control) and B (U251 MG MMP-1 over-expressor) are representative H&E sections. C (U251 MG control) and D (U251 MG MMP-1 over-expressor) are CD31 DAB immunohistochemical sections counter-stained with hematoxylin; red asterisk in D indicates a non-endothelial, ductile structure that is not immunoreactive for CD31. E, automated Ariol platform analysis of CD31 immunohistochemistry, where positivity is represented by percent of total region of interest area based upon multiple color channels and the morphology of immunoreactive structures (n = 3 tumors per condition, total of 9). *p<0.05.
were 22.29% positive (p<0.05, Figure 3.4E). These observations indicate that tumor produced MMP-1 was contributing to a significant recruitment of host nascent vascular structures within tumors in vivo.

**Angiogenic protein profile in glioma cells with respect to MMP-1 expression**

While MMP-1 clearly induced angiogenesis *in vivo*, and with *in vitro* modeling, the route(s) taken to do so remain unknown. For future studies it was sought to identify angiogenesis related proteomic changes that might occur in the context of shifts in MMP-1 expression. Glioma cell lysates from multiple passage points were pooled and applied to arrays spotted with 55 antibodies raised against factors known to be involved in angiogenesis. An inverse relationship between MMP-1 expression and the detection of certain anti-angiogenic proteins, such as endostatin and platelet factor 4 (CXCL4) was observed. However, most notable in these shifts was a substantial increase in the detection of TIMP-4 (Figure 3.5A&B) in the absence of MMP-1. There were no remarkable shifts in the detection of pro-angiogenic proteins with changes in MMP-1. This does not preclude the possibility that MMP-1 could induce other pro-
Figure 3.5. TIMP-4 expression with MMP-1 knock-down. A (T98G control) and B (T98G MMP-1 knock-down) present representative angiogenesis-related proteomic antibody arrays; circles denote the TIMP-4 duplicate antibody spots. C, representative MMP-1 and TIMP-4 immunoblot on T98G lysates to confirm by a resolved protein assay that with the knock-down of MMP-1 there is an increase in TIMP-4 immunodetection. D, densitometry on TIMP-4 immunoblots tested on different lysates (n =4 per condition). *p<0.05.
angiogenic mediators, *e.g.* protease activated receptor-1 (PAR-1), which were not included in these arrays.

**Confirmation of MMP-1 dependent TIMP-4 protein expression and anti-angiogenic role**

One of the sacrifices of high-throughput antibody arrays is an increased chance for off-target antigen detection, since the protein lysates are not resolved. It is well known that TIMPs can complex to other proteins with marked promiscuity. Therefore, traditional immunoblot analyses were performed to confirm changes in TIMP-4 protein levels. Glioma cell lysates were individually examined across multiple passage points, and a consistent increase in TIMP-4 detection in the absence of MMP-1 was observed (*p*<0.05, Figure 3.5C&D).

Recently, TIMP-4 has been reported to be a potent inhibitor of microvessel growth in an *ex vivo* aortic ring model of angiogenesis (Aplin *et al.*, 2009). Thus, the confirmation of these observations was sought with the microcarrier bead *in vitro* model. Both endothelial cell projection length and number were significantly reduced in the presence of rhTIMP-4 (*p*<0.05, Figure 3.6A&B). The combination of rhMMP-1 and rhTIMP-4 impeded projection length more so than TIMP-4 alone, while not significantly altering the number of projections per bead. Thus the anti-angiogenic properties of TIMP-4 were confirmed with the
Figure 3.6. In vitro angiogenesis assays in the presence of rhMMP-1 (10ng/L), rhTIMP-4 (50ng/L), and in combination. A, endothelial cell projection length. B, endothelial cell projection number. *p<0.05.
A  Endothelial Cell Projection Length with MMP-1 and TIMP-4

B  Endothelial Cell Projection Number with MMP-1 and TIMP-4
in vitro model, and its inverse relationship with MMP-1 expression, a traditional TIMP target, indicate a need for continued investigation.

III.5 Discussion

Glioblastoma is particularly insidious because of its ability to aggressively infiltrate the normal host parenchyma. Invariably this tumor cell diffusivity contributes to recurrence after treatment of the primary tumor. Therefore it is within reason to pursue the inhibition of pro-invasive factors such as the MMPs. It is known that MMP-1 is expressed in human GBM while not in normal brain, and that the knock-down of MMP-1 impairs GBM cell motility. However, it is also important to consider that MMPs are capable of much more than the clearance of ECM in a physical barrier context. Among the non-traditional substrates for MMP-1 are perlecan, insulin growth factor binding proteins, the pro-gelatinases (MMPs-2 and -9), and PAR-1 (Blackburn and Brinckerhoff, 2008). This implies a potential tumorigenic role for MMP-1 not just through the direct facilitation of invasion, but also via the activity enhancements of other pro-invasive enzymes and the release of mitogenic growth factor cues. Within this work it is reported that the expression of MMP-1 significantly influenced tumor incidence in in vivo. Increased levels of MMP-1 were consistent with
enhanced tumor take, as well as the size of tumors for all times examined.

More recently, the inhibition of VEGF has led to promising survival extension in recurrent GBM and is under trial with new diagnoses. However, detailed mechanisms are not entirely understood, and notable concerns have arisen regarding the physiology behind tumor responses (Verhoeff et al., 2009). The investigation of other tumor angiogenesis targets is needed and currently underway. Blackburn, Brinckerhoff, and colleagues (2007, 2008 & 2009) have reported compelling evidence that MMP-1 is a pro-angiogenic mediator in a melanoma setting through the activation of PAR-1 in a site unique from that of thrombin. Knowing that profuse tumor-associated angiogenesis is a hallmark of GBM pathology and with the knowledge that GBMs manufacture MMP-1 in an environment free of this protease, a similar effect was hypothesized. Three-dimensionally stabilized HUVECs were induced to proliferate and differentiate into sprouting, cord-like structures when co-cultured with GBM cells, while otherwise randomly migrating in an undifferentiated state; thus establishing a GBM cell induced in vitro angiogenesis model. Herein it was observed that the presence of MMP-1 significantly enhanced sprout extension in HUVEC-GBM cell co-cultures. When cultured without the GBM cells, but recombinant human MMP-1 alone, HUVECs were still induced to form sprouts. These data
complement others in the literature previously mentioned and confirm that MMP-1, at least in an in vitro setting, is involved in GBM cell induced angiogenesis.

The in vitro model relies on controlled and spatially distinct co-cultures thus this MMP-1 mediated angiogenic potential was assessed in vivo using U251 MG flank tumors via CD31 immunohistochemistry. There was an intense induction of CD31 immunoreactivity with MMP-1 over-expressing tumors versus controls. Endothelial structures were not just prevalent surrounding the tumor mass but invested the entire tumor interstitium more heavily than low expressing MMP-1 controls. Nearly 7-fold higher CD31 positivity was observed with MMP-1 expression, lending more evidence to the multi-faceted pathological roles that MMP-1 might fulfill in GBM. The contrast with respect to the scale of MMP-1 dependent changes between the in vitro and in vivo settings highlights the complex balance of angiogenic signaling events involved and gaps between in vitro vs. in vivo systems. We posit that MMP-1 is a major tumor produced factor contributing to the angiogenic switch characteristic of GBM (Tate and Aghi, 2009).

In light of our hypothesis and the aforementioned results, 55 angiogenesis related proteins were examined using commercially available antibody arrays. Strikingly, no substantial changes in pro-angiogenic factors were observed in
the context of MMP-1. We submit this as an additional emphasis of the complex balance between anti- and pro-angiogenic factors. Indeed, upon assessment of anti-angiogenic factors, several were found to have inverse detection with respect to the level of MMP-1 protein. The most remarkable of these changes was with TIMP-4, with its detection significantly higher in GBM cell lysates without MMP-1 expression. Therefore it is proposed that MMP-1 could be serving tumors by easing anti-angiogenic pressures.

It is widely accepted that all TIMPs are anti-angiogenic through the inhibition of MMPs that contribute to endothelial migration, halting endothelial cell cycle progression, and ablation of VEGF expression (Rege et al., 2005). However, there remains a dearth of literature with regard to TIMP-4, the newest member of that family. The most recent study displays evidence, from an aortic ring model, that TIMP-4 inhibits nascent vessel growth from the aortic slice, while stabilizing microvessels that already passed through an angiogenic cycle without TIMP-4 (Aplin et al., 2009). An earlier set of work confirmed the anti-migratory effect of TIMP-4 but reported no change in vessel formation in a chick chorioallantoic membrane model (Fernández and Moses, 2006). Thus TIMP-4 was applied to the HUVEC coated microcarrier bead assay and it was observed that rhTIMP-4 inhibits both the formation and organized growth of endothelial
projections. The two prior mentioned studies and this one, while having differences among them, all confirm at least one aspect of the anti-angiogenic property of TIMP-4; the points of contention could be attributed to the distinct methods among the models and the intrinsic theoretical practice of in vitro systems.

Matrix metalloproteinase-1 offers outstanding potential as a therapeutic target by at least three standards: it is unique to GBM with respect to the local host parenchyma, it is pro-angiogenic, and it is pro-migratory and invasive. Unfortunately in the past decade, the use of a MMP non-specific inhibitor in a phase II trial resulted in intolerable joint pain side effects (Groves et al., 2002). Current MMP inhibitors have broad effects, and potential MMP substrates remain difficult to predict; these factors underline the need for an assemblage of structural biology investigation and expertise into these enzymes. Furthermore, the interplay of MMP-1 with anti-angiogenic proteins as discussed in this work could be exploited for clinical value and warrants extensive continued exploration. The inverse relationship between MMP-1 and TIMP-4 is particularly intriguing in light of the generally accepted principal that TIMPs inhibit MMPs; the discovery of the mechanisms underlying this relationship will be of exceptional scientific value.
Chapter 4

Discussion
IV.1 Summary of Thesis Results

Herein is outlined experimentation into the important roles of MMP-1 in GBM biology. The first set of experiments set out to examine the potential stimulation of MMP-1 protein production by the small bioactive diatomic free radical NO. The rationale behind this can be set forth by at least three relevant points: first, the expression of the enzymes that produce NO, the NOS, is higher with increasing grade of astrocytoma, peaking in GBM; second, this profile overlaps with a locally unique over-expression of MMP-1 in patient samples (Table 1.3; McCready et al., 2005); and third, NO is known to positively influence multiple signal transduction pathways that are aberrantly amplified in the GBM condition, of which, specifically the MAPK cascades, the MMP-1 promoter is exquisitely sensitive.

With the use of NO donors at a concentration that reflects the production of cNOS, significant increases in MMP-1 mRNA and protein were observed indicating a causal expressive link. However, there remained a question of function. Application of the NO donors significantly enhanced glioma cell motility in vitro. However, more important was the finding that with the transient inhibition of MMP-1 protein production with RNA interference technology, there was a substantial abrogation of the NO-induced migratory response.
This is a key point of study since one of the most difficult aspects of GBM treatment is the inability to access small populations of tumor cells that migrate away from the surgically resectable tumor mass. Eventually these migratory cells, intimately interspersed throughout the host parenchyma, cause recurrent lesions that lead to death. Matrix metalloproteinase are integral components of cellular motility and invasion. It is important to note that MMP-1 is not the only MMP with proven pathological activity in GBM, but what makes it an attractive candidate is the fact that its expression is profoundly unique to the tumor and that it possesses the ability to liberate other factors and MMPs, specifically the gelatinases which have been well-studied with respect to GBM cell invasion. Targeting the activity of NOS is promising because of the vast knowledge on signaling systems and plethora of pharmacological inhibitors that are available for pre-clinical study. Indeed, within this body of investigation (Chapter 2) it was observed that the use of one of these inhibitors, L-NAME, significantly reduced GBM cell migration.

The second portion of this work focused upon the importance of MMP-1 in GBM with explicit attention to tumorigenic and angiogenic pathologies. Glioblastoma cells are particularly hardy in the non-permissive host environments – by exploiting a poorly understood complex arrangement of cell survival, matrix
sensing, and degrading signals GBM cells are able to settle, reproduce, and spread through normal host tissue. Furthermore, they secrete factors that lead to the profuse production of leaky and sometimes non-circulating blood vessels. The bases of these pathologies are not completely understood and require continued investigation. As such models of tumorigenicity and angiogenesis were examined in the context of MMP-1 expression.

Two GBM cell lines were engineered to stably over-express or inhibit MMP-1 protein production depending upon their parental cell phenotypes. These MMP-1 modified cells were then grown subcutaneously. It was observed that GBM cell tumorigenicity was significantly dependent upon MMP-1 expression status, which was confirmed with MMP-1 immunodetection in tumor lysates. In the presence of MMP-1, the formation of palpable tumors was approximately twice that in cells without the enzyme. Furthermore, the sizes of tumors that formed in groups with lower MMP-1 expression were half the volume of their respective counterparts.

Viability data from the NO experiments and as yet unpublished work by Anand and colleagues (2010) show that MMP-1 does not directly enhance GBM cell proliferation in vitro, nor does it impede survival or cellular reproduction. These collective data suggest two possibilities to account for increased tumorigenicity that likely overlap in an in vivo
setting. First, as mentioned previously, there exists a multitude of non-traditional MMP-1 substrates in the host interstitium, which upon activation or release by MMP-1 proffer widespread effects on not just invasion but also growth and resistance to apoptosis. Second, MMP-1 might not be as directly important for proliferation but serves as a survival factor when GBM cells are introduced to or sense the non-permissive host setting, which is not replicated in vitro.

Following demonstration that GBM cell tumorigenicity was significantly enhanced by the presence of MMP-1, the impact of this enzyme upon the other GBM pathology of angiogenesis was sought. An in vitro model of tumor cell induced angiogenesis in a three-dimensional environment indicated that MMP-1 and especially GBM cells expressing MMP-1 were capable of inducing an in vitro angiogenic phenotype by extending endothelial sprout length and increasing sprout number. Subsequently, excised tumors from the in vivo tumorigenicity experiments were assayed for reactivity with a host specific endothelial factor, CD31, and subjected to quantitative IHC analysis, which revealed a marked increase in nascent endothelium within tumors, further indicating a pro-angiogenic role for MMP-1.

Only recently has MMP-1 been suggested to fulfill a pro-angiogenic function. Blackburn, Brinckerhoff and colleagues posit that this is, in part, through the activation of a GPCR
known as PAR-1. However, the interplay of other proven angiogenic factors remains elusive. As such the angiogenic proteomic profiles of GBM cells in the context of MMP-1 expression were examined. Strikingly, notable changes in the detection of other pro-angiogenic proteins were not observed. However, in the absence of MMP-1 there were increases in anti-angiogenic factor detection, the most profound of which was TIMP-4. This inverse relationship with TIMP-4 was additionally confirmed, with immunoblot analyses, as well as its anti-angiogenic activity in the in vitro model. Since it is the most recently discovered member of its family, comparably little is known about TIMP-4, aside from MMP inhibition and anti-angiogenic capabilities. This relationship of TIMP-4 with one of its alleged inhibitory targets raises the intriguing question of whether the MMP is controlling the TIMP or vice versa, especially in light of TIMP-2 facilitated MMP-2 activation.

Altogether these data confirm that MMP-1 is an important mediator of GBM pathology, and not just an expression phenomenon of these tumors. Based upon the various processes it positively influences in gliomagenesis, MMP-1 is an attractive target for molecularly focused therapeutics. It will be of importance to study various approaches for inhibiting the pathological activity of MMP-1, for example by means of the inhibition of
upstream mediators such as NO, or the direct inhibition of the enzyme itself.

### IV.2 Future Studies

To date there have been few studies reporting on MMP-1 in GBM. Until the current work these investigations focused on expression patterns, where for example in Hodgson et al. (2009) MMP-1 was one of many conspicuously over-expressed proteins in GBM that came about from various array and gene copy analyses. Prior work from our laboratories by McCready and colleagues (2005) highlighted the importance of a single nucleotide polymorphism in the MMP-1 promoter (2G) in its association with GBM and ability to drastically increase MMP-1 expression. This allele was shown to increase the sensitivity of the MMP-1 promoter to the MAPK signal transduction pathways by introducing a secondary Ras Responsive Element. This provided further illustration, since Rutter et al. (1998), of the importance of the ETS and activator protein (AP) families of transcription factors in mediating MMP-1 transcription. Stojic and colleagues (2008) were able to confirm the expression observations of McCready where there was no detectable MMP-1 transcript in either normal brain or low-grade astrocytoma. They were the first to correlate MMP-1 with the WHO grading of astrocytomas,
GBM having the highest mRNA and protein expressions over any lower grade samples.

The current work is the first to examine the function of MMP-1 in the context of GBM. Based upon the results of the studies within this thesis and the prior expression studies of others, one can construct the general mechanistic pathways outlined in Figure 4.1. It becomes clear that while our knowledge of MMP-1 expression and function has improved over the past decade, there are still a number of basic scientific questions that require exploration.

First with regard to the control of expression of MMP-1, while it is well established through the works of the Brinckerhoff, Fillmore, and Esumi (who studied NO stimulation of p38 and ERK) laboratories that the MAPK pathway is the prime mediator behind MMP-1 expression, it is still unknown which ETS family members are responsible in any cancer condition. Several investigators have reported on this issue, and note that a continuing burden of limited antibody use must be overcome for efficacious chromatin immunoprecipitation and electrophoretic mobility shift assays to proceed. It has been shown from these investigations however, that there are various ETS transcription factors highly expressed in neoplastic tissue, which is fitting to the model that Brinckerhoff posits, and with which McCready concurs: that the MMP-1 promoter is at-the-
Figure 4.1. Theoretical pathways of MMP-1 significance in glioblastoma cells. It is known through the work of multiple investigators that MMP-1 expression is particularly dependent upon the activity of MAPK pathways. McCready et al. confirmed an enhanced expression with the introduction of an additional ras responsive element in GBM cells. Ishii et al. (2003) demonstrated that NO can induce the activity of p38 and ERK kinases (MAPK) leading to downstream induction of MMP-1 transcription in melanoma. It appears that MMP-1 can somehow ease anti-angiogenic factors such as TIMP-4, CXCL4, and Endostatin, by unknown mechanisms. However, it is also possible that MMP-1 could be directly pro-angiogenic and mitogenic through the activation of PAR-1 and the release of ECM-sequestered growth factors.
ready in cancer, but requires the aberrant activation of its constituent transcription factors. Furthermore, repression of MMP-1 transcription by p53, presumably by interfering with other transcription factors (Sun et al., 1999) has elicited interest and is under investigation in our laboratories, especially in the context of ‘gain-of-function’ mutant p53 isoforms.

Nitric oxide, with its source enzymes correlated with increasing glioma grade as discussed in chapter 1, and functional impact (chapter 2), is likely one of the factors responsible for an increased presence of MMP-1 in GBM. Therapeutics related to NO remain attractive (discussed in the following section), specifically because of the number of NOS inhibitors available, but as with the elusive ETS transcription factors, the relevant technology for better understanding NO in GBM is limited. It is still unknown precisely how much NO circulates in these tumors, and there is scant evidence regarding NOS activity. Nitric oxide is notoriously difficult to work with because of its short lifetime. Indeed, seeking an effective methodology for tissue detection of NO has evolved into a field unto itself, and seeking answers in regard to GBM will require extensive collaboration with NO physiologists, and the development of stable NO probes for the CNS.

Beyond expression concerns, the matter of NO emphasizes the importance of studying MMP-1 activity. As mentioned previously,
in a seminal study Gu and colleagues (2002) demonstrated the permanent activation of MMP-9 through a high-NO-concentration dependent post-translational mechanism termed S-nitrosylation. Specifically, complete removal of the MMP-9 pro-domain was facilitated by S-nitrosylation of the conserved, critical ‘cysteine switch.’ Such a prospect in MMP-1 is enticing. Efforts of this author to identify S-nitrosylation of MMP-1 proved fruitless, but do not preclude the possibility, as just the detection of S-nitrosylation remains a contentious issue. While this offers an interesting proteomic theory, the high levels of NO needed to consistently S-nitrosylate pro-MMPs might not be locally sustained in GBM, which again highlights the importance of NO detection first.

An additional interesting finding from Stojic et al. (2008) was the detection of MMP-1 in GBM tissue at a molecular weight consistent with active enzyme (42kDa) concomitant with pro-MMP-1 (52kDa). When lysates from tumors grown in vivo in this work were analyzed (Figure 3.2) immunoreactivity was suggestive of the active species of MMP-1, which could account for the lack of impact upon glioma cell growth in vitro, yet significant increases in tumorigenicity in vivo. Targeting the active enzyme through hydroxamate zinc chelation has governed the past research of MMP therapy (discussed in IV.4). Determining the impact on pathological function due to relative levels of active
MMP-1 is an important aspect for future investigation. To concretely explore this prospect an effective activity assay will need to be applied. Several vendors offer sensitive FRET based substrate assays, however these are not specific to MMP-1 when assessing tissue specimens. Blackburn et al. (2007) described an interesting in vitro collagen gel based evaluation, where gel weight before and after the application of active MMP is assessed. The implementation of such an assay to activated MMP-1 over-expressers could provide preliminary insights.

An exciting finding in this work was the functional implication of MMP-1 expression on GBM cell-induced angiogenesis. How is this happening? To the author, this is the most intriguing basic scientific question. As detailed earlier (chapters 1 and 3), the Brinckerhoff laboratory is currently working toward a model where MMP-1 activates the pro-angiogenic receptor PAR-1. An unexpected finding through the proteomic arrays in chapter 3 was that none of the included pro-angiogenic factors were reliably altered with respect to the status of MMP-1 expression. Instead, anti-angiogenic factors appeared to be stimulated in the absence of MMP-1, more surprising was the degree to which this disposition was true with TIMP-4, a traditional MMP inhibitor. As the newest member of the TIMP family, there is little functional data available for TIMP-4. What seems to be certain is that it is anti-
angiogenic (chapter 3; Aplin et al., 2009), yet the mechanism for this activity is unknown. The potential abilities of TIMP-4 are largely assumed based upon other TIMPS, but for example, TIMP-2 is well known to facilitate the activation of MMP-2. Therefore, such assumptions are not tenable. It would be valuable to continue studies examining the expression of TIMP-4, especially in patient GBM samples, in a more extensive, rigorous manner. To date, Groft and colleagues (2001) provide the only report of TIMP-4 in glioma; mRNA expression was examined in eight tumors, only two of which were high-grade. Their data suggests that TIMP-4 expression correlates negatively with tumor grade, but requires further validation and functional investigation. Techniques similar to those used herein – stable over-expression and knock-down – could provide the tools necessary to gain more insight into the role of TIMP-4, certainly in light of its relationship with the MMPs.

The final question would be one of how MMP-1 can affect TIMP-4 expression. The data presented in chapter 3 suggest that MMP-1 could be inhibitory toward anti-angiogenic factors. One might hypothesize that a direct interaction between the two proteins not only inhibits further MMP-1 activity, but also negates TIMP-4 anti-angiogenic activity. However in the face of the expression data in chapter 3, a direct post-translational event acting upon TIMP-4 seems unlikely. Could MMP-1 be active
intracellularly to influence TIMP-4? Supportive literature for such a hypothesis is certainly available. The activation of the MT-MMPs and MMP-11 and -27 through furin cleavage has been observed within the cell (Hockenbery, 2006). Si-Tayeb and colleagues (2006) reported the potential for MMP-3 to localize to the nucleus; Kwan et al. (2004) demonstrated this for MMP-2, which could also cleave PARP. In fact a relevant investigation proffered by Limb and coworkers (2005) illustrates *in vitro* the capability of MMP-1 to localize to the mitochondria, and potentially provide resistance to apoptosis by preventing lamin A degradation and caspase activation. Exploring intracellular possibilities with MMP-1 in GBM cells might provide information on this paradoxical MMP-TIMP relationship.

**IV.3 The State of NO Targeting in Cancer Therapeutics**

The roles of NO production in cancer pathophysiology are rather paradoxical and as such methods for exploiting NO or its parent enzymes the NOS remain one of the most hotly debated subjects in cancer research. It is now generally accepted that the observably dichotomous effects of NO are concentration dependent. In general lower levels of NO, less than micromolar, are tumorigenic while higher concentrations are tumoricidal. Early studies into the prospects of NOS inhibition in cancer were focused on the anti-angiogenic potential, as well as reduction of tumor specific blood circulation. In the melanoma
and adenocarcinoma settings, the same arginine analog (L-NAME) used in the aforementioned sections selectively inhibited tumor blood flow (Andrade et al., 1992). This was later replicated in rats harboring P22 carcinosarcomas (Tozer et al., 1997). This effect was reversible in all cases with the administration of L-arginine, the substrate for NOS activity. Furthermore, there was a marked dose-dependence, that once the optimal dose of NOS inhibitor was surpassed, no further reduction in tumor blood flow was seen. This is attributable to one of the problems with the systemic administration of NOS inhibitors: increase in mean arterial blood pressure, i.e. hypertension.

Closer examination of the impact of L-NAME administration with the goal of restricting tumor induced nutrient flow showed that the inhibitor can act synergistically with other anti-tumor treatments such as melphalan, TNF-α, and IL-2 (Orucevic and Lala, 1996; de Wilt et al., 2000). Both of these works were conducted in adenocarcinoma models, where Orucevic and Lala present data that the inhibition of NO production assists IL-2 mediated toxicity. The production of anti-tumoral lymphokine-activated killer cells (LAK) is assisted by IL-2; normally, NO inhibits the proliferation and activity of LAKs, and thus NOS inhibition improved IL-2 mediated tumor resolution. In a later elegant study, Okada and colleagues (2006) observed a vital role for NO in cancer progression. Benign murine fibrosarcoma cells
were grown in vivo in gelatin sponge grafts (to induce an inflammatory response). An iNOS specific inhibitor, aminoguanidine (AG), was systemically administered through drinking water to the experimental arm of the study. Tumors +/- AG treatment were harvested, homogenized, and intravenously administered to syngeneic mice to assess metastatic potential. Both the incidence of metastasis and number of metastatic nodules in the lungs of mice with AG treated tumors were substantially reduced, indicating that NO was vital to the malignant transformation of this tumor type. Metastatic, tumorigenic, invasive, and motile potentials of NO are supported by these studies, Ishii and coworkers (2003) showing the induction of specific MMPs in melanoma, and the present work demonstrating the pathological contributions of MMP-1 in glioblastoma.

Despite the clear evidence that NO, at levels presumably produced by a tumor and through the use of NOS inhibitors, is a tumorigenic molecule, there remains a substantial force in research for the use of excessively high concentrations of NO and its related nitrosative stress products, e.g. peroxynitrite, as tumoricidal agents. One such suggestion has been through the lens of bioreductive chemotherapeutics. These drugs target hypoxic tumor areas where they are reduced into cytotoxic metabolites. In the context of iNOS over-expression, it has
been shown that the DNA damage induction of at least one of these drugs, Tirapazamine, is potentiated presumably by P450 reductase, to which the NOS enzyme structures are similar (Saunders et al., 2000; Chinje et al., 2003). While the use of hypoxia targeting chemotherapeutics is useful, especially considering recent evidence supporting the HIF-2α axis in multiple human cancers (Franovic et al., 2009), the relevance of these drugs to NO in highly invasive, oxic cancer cells, such as in the periphery of glioblastoma, is not concrete.

Most approaches to using high levels of NO as a treatment involve the systemic delivery of NO donors. However, unlike the NOS inhibitors which have shown significant and specific antitumoral effects at less than systemically detrimental doses as aforementioned, the therapeutically meaningful doses of NO required from most donors in a glioblastoma setting are impractically high, i.e. on the order of hundreds of micromolar to tens of millimolar (Weyerbrock et al., 2009). This produces the immediate and obviously life threatening effect of profound hypotension (Abrams, 1992) before mechanisms of cytotoxicity, which require temporally extensive administration, can even become manifest. Attempts have been made to circumvent this issue, such as the indirect activation of iNOS through IFN-γ, or iNOS based, hypoxia driven gene therapies, but the extensive
side effects or low efficiencies of these approaches has limited continued investigation (Fitzpatrick et al., 2008).

**IV.4 The State of MMP Targeting in Cancer Therapeutics**

The by and large agreement in the literature that a number of the MMPs are integral to tumor cell migration, invasion, and metastasis made them attractive targets for direct inhibition starting in the 1990’s. An early inhibitor known as batimastat (BB-94) is a synthetic peptide that mimics a collagen cleavage site, thus making it a broad spectrum MMP inhibitor. Its inhibition is dependent upon a hydroxamate moiety that chelates the MMP active site zinc atom, thus blocking hydrolysis, however the details regarding molecular interactions behind hydroxamate-inhibitor specificity remain incomplete (Brown and Giavazzi, 1995; Jani M et al., 2005). The use of this inhibitor has shown a reduction in metastatic tumor burden in murine melanoma models, but no cytotoxic impact (Chirivi et al., 1994; Brown and Giavazzi, 1995). Furthermore, the progression of human ovarian and colorectal carcinomas as well as murine hemangioma growth and angiogenesis were inhibited (Brown and Giavazzi, 1995).

Batimastat was taken to phases I and II clinical trials, and subsequently it was discovered that oral dosage of the drug did not allow efficacious bioavailability. Intraperitoneal administration was required to have noticeable effect, however
this method is an undesirable treatment modality (Brown and Giavazzi, 1995; Wojtowicz-Praga et al., 1997). Therefore, a second generation of MMP inhibitors with oral bioavailability was produced, of which one known as marimastat (BB-2516) was championed. It too is a collagen-like peptidomimetic with a zinc-chelating hydroxamate. It was reported to have specific activities against MMPs-1, -2, -3, -7, and -9, especially MMPs-1 and -9 (Wojtowicz-Praga et al., 1997). Pre-clinical results for the use of marimastat were similar to those of its predecessor (Wojtowicz-Praga et al., 1998). Additionally, studies were conducted describing significant reductions in glioblastoma cell invasion and in vitro tumor growth with both hydroxamate-based inhibitors. This effect was evaluated for impacts on cell viability and it was observed (again for batimastat but with novelty for marimastat) that the anti-tumor activity of these drugs was predominantly through cytostatic, not cytotoxic, mechanisms, which made marimastat an even more promising drug candidate in the context of preserving host CNS tissue (Tonn et al., 1999).

However, the enthusiasm for the second generation MMP inhibitor soon began diminishing. A phase I trial in advanced lung cancer patients demonstrated the oral efficacy of marimastat delivery through the gut, however a dose limiting inflammatory polyarthrits toxicity was observed, which had a
cumulative effect because of lingering plasma drug concentrations. Since plasma concentrations that replicated the in vitro anti-tumoral effects of marimastat were achievable at low oral dosage it was recommended that its use be further investigated (Wojtowicz-Praga et al., 1998). A phase II study of marimastat plus temozolomide in recurrent GBM patients showed a progression free survival benefit. However nearly half of the patients experienced drug-induced arthritis, most often in hand joints, and the study population was too small for any rigorous statistical evaluation (Groves et al., 2002). Other human data, especially in metastatic breast cancer patients, practically removed marimastat from anti-cancer consideration. A prominent phase II trial showed that marimastat did not extend progression free survival in breast cancer patients, but rather elicited the same musculoskeletal toxicities mentioned before, which were actually associated with reduced survival (Sparano et al., 2004). These data in combination with a perceived ill-placed pre-clinical optimism for batimastat and marimastat led to pariah status and eventual downfall of the drugs’ patents holder British Biotech. More extensive clinical data regarding the use of marimastat in glioma were slower to evolve, but did not offer any enthusiastic results, where no benefit over temozolomide alone was observed in recurrent anaplastic astrocytoma (Groves et al., 2006), and in a larger double-blind, placebo controlled
GBM study, no progression free survival benefit was recorded (Levin et al., 2006).

Despite this hit to the study of MMPs in cancer pathophysiology, there remain other promising targeting avenues. Another hydroxamate-based inhibitor, prinomastat (AG3340), demonstrates particular selectivity for the MT1-MMP mediated invasion axis (Scatena, 2000). It also shows potency toward MMP-13, another collagenase recently found over-expressed in malignant glioma. Indeed, comparably little work has been published regarding prinomastat, likely because of the distaste associated with hydroxamate MMP inhibitors, but one extensive pre-clinical study reported profound inhibition of GBM cell proliferation, in vivo tumor growth (s.c. model), and a long lasting, host-positive effect (Price et al., 1999). A clinical pharmacokinetic study of this inhibitor in multiple advanced stage cancers found that it could elicit the same undesirable side effects of its hydroxamate predecessors, but these issues were only seen with excessive dosing while lower doses achieved desirable plasma concentrations without deleterious side effects (Hande et al., 2004). However, a subsequent phase III study reported no chemotherapeutic benefit with prinomastat in NSCLC (Bissett et al., 2005). However, as mentioned earlier in this work (Chapter 1) at least with regard to EGFR therapies, there are considerable differences in effectiveness of treatment.
modalities between NSCLC and GBM. A phase II GBM study was completed in 2002, however the results have yet to be reported, and presumably its use in GBM is still under investigation (Adamson et al., 2009).

Naturally occurring macrocyclic lactides have also been proposed for investigation in malignant gliomas (da Rocha et al., 2002). Bryostatin-1 is a compound of specific interest, which is isolated from the larvae of Bugula neritina, a marine bryozoan. This compound has been shown to induce the differentiation of cancer cells in vitro by activating protein kinase C (PKC) after early exposure, while eventually leading to a reduction in the kinase, which notably inhibits the production of MMPs-1, -3, -9, -10, and -11 but does not alter their activities (Wojtowicz-Praga et al. 1997; Johnson et al., 1999). Recent clinical data using bryostatin-1 as a specific agent show mixed, tumor specific responses. For example, Lam and colleagues (2009) report that the combination of bryostatin-1 and paclitaxel is not an effective paradigm for advanced pancreatic carcinoma. However, Barr and coworkers (2009) observed efficacy in certain cases of aggressive B-cell non-Hodgkin lymphoma. It has been noted by both investigators that newer, more effective and specific ‘bryologs’ are in development that might show improvements upon bryostatin-1 in the clinic.
The potential for bryostatin-1 and its analogs remains to be studied in GBM and many cancer conditions altogether.

The toll-like receptor 9 (TLR9) sub-family of TLRs are intracellular components of the immune system. The traditional ligands of TLR9 are unmethylated CpG areas of microbial nucleic acids. Binding and activation of these receptors is commonly described as leading to the production of various inflammatory cytokines (Wagner, 2004). Recalling the promoter structures of the MMPs described in Chapter 1, it is reasonable to theorize that the downstream transcription factors up-regulated by TLR9 activity, such as NfκB, could also target and elicit MMP gene transcription. Merrell and colleagues (2006) described MMP-dependent induction of invasion by TLR9 CpG-oligonucleotide (ODN) agonists. These compounds mimic the microbial ligands of TLR9, thereby causing their activation. The use of another MMP inhibitor, ilomastat (GM6001), or MMP-13 specific antibodies abrogated CpG-ODN instigated invasion in GBM and breast cancer cell lines. More recent work by Meng et al. (2008) describes the expression of TLR9 in patient GBM samples as largely immune cell localized, in agreement with Merrell’s assertion that infectious responses by immune system components might contribute to cancer progression.

Finally, the excitement surrounding anti-angiogenic therapies for GBM, specifically bevacizumab, brings to light
once again the importance of MMPs. A current publication presents evidence that the treatment of GBM cells with bevacizumab alters the expression of several of these enzymes. The expression characteristics are not the same across MMPs, where MMPs-2, -9, and -12 were stimulated by bevacizumab, and interestingly educed a 25-fold reduction in MMP-1. Furthermore, it was posited that bevacizumab resistant cells utilize over-expression of at least MMPs-2, -9, and -12 as well as TIMP-1 to subvert the therapy (Lucio-Eterovic et al., 2009). These findings, especially the implication of TIMP-1, are especially prescient in light of this body of work.

**IV.5 Concluding Remarks**

Glioblastoma multiforme has evaded the best-focused efforts of neuro-oncologists for decades. While it is a relatively uncommon cancer, diagnosis with GBM is, with near certainty, a fast-attacking, terminal disease. Few other conditions can have as an immediately detrimental and seemingly helpless impact in the manner that GBM can. Notwithstanding this dire history there have been some steps in treating GBM. The front-line effort of tumor de-bulking has vastly improved with neurosurgical techniques, equipment, and thinking with a firm emphasis on removing as much of a primary tumor mass as
possible. Newer imaging techniques, for example through the use of metallofullerene caged contrast enhancement agents currently under investigation in our laboratories, have a strong potential to assist neurosurgeons in more confidently achieving the greatest possible extent of resection. In addition, radiation therapy modalities and temporal paradigms have been focused and optimized over the years and now work in concert with chemotherapeutics such as BCNU and temozolomide to improve median survival.

However, gone are the days of the exclusive use of grossly non-specific therapies. Molecular targeted therapeutics is the new front of treating this and other diseases. With the improvements in tools, and knowledge, e.g. genomes, and proteomes, and experience, efforts attacking specific genes and proteins offer promising avenues for combinatorial therapies. The swift evolution of anti-VEGF therapies in addition to traditional modalities in multiple advanced cancers including GBM is a prime example of this movement. Many clinicians and researchers, including this author, envision a not-so-distant future when many afflictions like GBM will have individual patient tailored remedies and specifically targeted drug approaches.

The field of MMP research in cancer undoubtedly suffered from disappointing inhibitor studies at the turn of the 20th and
21st centuries. However, these inhibitors were based on a singular (collagen mimetic, hydroxamate) approach, with a lesser understanding of how MMPs were controlled. Today we know a great deal more about specific MMPs with regard to certain conditions, as presented for instance in this work, and potential upstream and downstream regulators and effects. Control of NO is one such promising path considering the preponderance of cNOS expression in high-grade tumors, and the availability of a plethora of NOS inhibitors. However, this will not be the only approach. Investigations are ongoing with regard to upstream and downstream regulation of MMP-1, for example the activation of PAR-1. Finally, it is the opinion of this author that it might be possible for the direct inhibition of MMPs in cancer to return to light. This will require the collaborative efforts of clinicians, pharmacologists, molecular, and structural biologists to discover effective small molecule or antibody therapies to combat this grim affliction.


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Hernandez-Barrantes S, Toth M, Bernardo MM, Yurkova M, Gervasi DC, Raz Y, Sang QA, Fridman R. 2000. Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to


Limb GA, Matter K, Murphy G, Cambrey AD, Bishop PN, Morris GE, Khaw PT. 2005. Matrix metalloproteinase-1 associates with


Appendices
Appendix A. Glioma Cell Scratch Motility Test

Purpose: To assess the No-induced motility of glioma cells.

Method: U-87 MG cells were passaged into 100mm diameter cell culture dishes at a density of $1.5 \times 10^6$ cells per dish. Dishes were divided between two conditions — either control (normal culture conditions as described in Chapter 2) or with 100µM SNP. The cells were allowed to settle and acclimate overnight. The next morning media were changed and three scratches were made in each dish using a 200µL pipette tip made for Rainin LTS pipettors. Images were immediately taken at 200x magnification phase contrast at three points along each scratch as a zero hour time point. Subsequent images were captured three and six hours later. The experiment was repeated thrice. ImageJ was calibrated to the appropriate magnification and utilized to calculate the area of scratches in square microns. Data are reported as percent decrease in scratch area.

Results: The inclusion of SNP in culture media significantly increased scratch closure by 16% ($p<0.01$) at the three-hour time point (figure A.1). Cells traversed scratches regardless of SNP by the six-hour time point. This type of experiment confirms the findings of the membrane based invasion assay. It should be noted that two of the major differences are: this assay requires nearly 100% confluence, and it is two-dimensional, whereas the membrane based assays test cells along a vertical axis.
A.1

Glioma Cell Scratch Motility Test

[Images of cell cultures at 0 hours and 3 hours for control and SNP treated groups]

[Graph showing percent decrease in scratch area for control and SNP treated groups]
Appendix B: pIREs and pLKO.1 vector maps

Since the alteration of MMP-1 for extended periods of time was an objective in Chapter 3 with the in vivo tumor growth and the in vitro angiogenesis assays, stable transfections were needed. Furthermore, while in theory transient transfections could have sufficed for the in vitro angiogenesis experiments, the stress of transient transfection methods, combined with the elaborate conditions of three-dimensional culture would likely have confounded the results. Finally, the consistency of using the same, pooled clones for the in vivo experiments was ideal. A standard cDNA system was utilized for the over-expression MMP-1; the first figure (B.1) is a map of the pIREs vector developed by the VCU-Massey Cancer Center Molecular Biology Core Facilities, which elicits forced expression of MMP-1 under the CMV promoter and can be stably selected for via neomycin (G418) resistance (map provided by Monika Anand). For the knock-down of MMP-1 production, a lentiviral system (second figure, B.2, modified from the Sigma MISSION® shRNA literature library) was chosen, where stable clones could be selected via puromycin resistance. Specific lentiviral particles were utilized containing sequences used for naked MMP-1 siRNA in the Chapter 2 methods.
B.2

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<th>Description</th>
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<td>cppt</td>
<td>Central polypurine tract</td>
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<tr>
<td>hPGK</td>
<td>Human phosphoglycerate kinase eukaryotic promoter</td>
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<tr>
<td>puroR</td>
<td>Puromycin resistance gene for mammalian selection</td>
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<tr>
<td>SIN/LTR</td>
<td>3' self inactivating long terminal repeat</td>
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<td>f1 ori</td>
<td>f1 origin of replication</td>
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<tr>
<td>ampR</td>
<td>Ampicillin resistance gene for bacterial selection</td>
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<tr>
<td>pUC ori</td>
<td>pUC origin of replication</td>
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<tr>
<td>5' LTR</td>
<td>5' long terminal repeat</td>
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<tr>
<td>Pse</td>
<td>RNA packaging signal</td>
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<td>RRE</td>
<td>Rev response element</td>
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Appendix C: Angiogenesis Antibody Array Summary

Whole representative, developed angiogenesis antibody arrays that were used in Chapter 3 for T98G cells are displayed in figure C.1; arrows indicate the location of TIMP-4 immunodetection. Table C.2 is a summary of percent change in the MMP-1 knockdown group compared to control. Figure C.3 is a graphical representation of notable changes other than TIMP-4. It should be noted that immunodetection for ADAMST-1 was low and variable across repetition, and that none of the changes seen in any other proteins approached the order of magnitude and consistency to which TIMP-4 is altered. The dark spots in the corners of each array are positive controls for loading.

Figure C.4 shows an angiogenesis antibody array conducted using pooled U251 controls and over-expressers. The differences in proteomic profiles between the two cell lines are apparent, as U251 expresses far fewer angiogenesis related proteins. Unlike T98G, U251 does not normally express MMP-1, or TIMP-4 as can be seen in C.4. These data lend evidence to a complex balance between pro- and anti-angiogenic forces, of which MMP-1/TIMP-4 are parts. Differences that were detected with forced MMP-1 expression were minor in comparison to T98G TIMP-4 data, failing to achieve fold changes; effective antibodies for confirmatory immunoblots will be needed for future studies.
C.1

T98G Angiogenesis Antibody Arrays

Control

shMMP-1
### Table C.2

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<td>TIMP-4</td>
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C.3 Notable Protein Expression Shifts with Stable MMP-1 Knockdown (other than TIMP-4)
Arrows denote the locations of the TIMP-4 antibody spots.

Noticeable changes included a reduction in the detections of endostatin (anti-angiogenic) and bFGF (angiogenic) with forced MMP-1 expression.
Appendix D: P-Kinase Antibody Array Summary

In an effort to assist future studies in identifying possible cell signaling factors sensitive to MMP-1 expression, antibody arrays (ARY003, R & D Systems) spotted against a limited number of phospho-kinases, i.e. active kinases, were conducted. These arrays were performed according to the manufacturer’s protocol, and lysates collected as described in Chapter 3 Methods. Figure D.1 displays representative T98G arrays. Table D.2 is a summary in the same fashion as table C.2, and figure D.3 is a graphical representation. The most consistent change in T98G MMP-1 knockdown lysates was an increased activation of Akt at threonine 308, denoted by asterisks in figure D.1; otherwise noted changes were for the most part subtle in terms of immunoreactivity and variable across repetitions.

Figure D.4 displays U251 P-kinase arrays. The most notable differences were decreases in all phospho-p53 (three pairs of descending spots below the black arrows in D.4) and p-CREB (‘+’ on the left side of the arrays). Interestingly there were converse changes in detection such as with β-catenin and CREB increasing with MMP-1 knockdown in T98G (Figure D.3), and decreasing with MMP-1 over-expression (Figure D.6). However, caution should again be exercised, as all these differences are subtle in the context of the control spots (again, the immunoreactive spots in the corners of the arrays). These data
remain inconclusive and are currently, in part, under investigation by Anand and colleagues.
D.1

T98G Phospho-Kinase Antibody Arrays

Control

shMMP-1
Table D.2. T98G p-kinase antibody arrays

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D.4

U251 Phospho-Kinase Antibody Arrays

Control

MMP-1+
<table>
<thead>
<tr>
<th>Name</th>
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VITA

Nicholas Alexander Pullen, a citizen of the United States of America, was born October 8, 1983 in Charlottesville, Virginia. He graduated from Jefferson Forest High School in Forest, Virginia, June 2001. He subsequently graduated with a Bachelor of Science degree, majoring in biology, from James Madison University in Harrisonburg, Virginia, May 2005. During his undergraduate years Nicholas worked as an assistant for the Department of Biology genetics teaching laboratories. He was also an undergraduate researcher who, under the tutelage of Dr. Terrie K. Rife, studied the prevalence of nucleotide polymorphisms in the neuronal nitric oxide synthase gene promoter in patients diagnosed with Parkinson’s Disease. Later in 2005 he joined the Department of Anatomy & Neurobiology at Virginia Commonwealth University where he completed his Ph.D. in May 2010, investigating the influences of matrix metalloproteinase-1 expression on glioblastoma pathology under the mentorship of Dr. Helen L. Fillmore. During the course of his doctoral studies Nicholas was recognized with the Jack Denning Burke Award for excellence in cell biology research. His work has produced multiple peer-reviewed publications regarding both nitric oxide and matrix metalloproteinase-1, with another manuscript under review as of Spring 2010, as well as numerous local, and national level conference abstracts. In June 2010 he will begin his postdoctoral work in the laboratory of Dr. John J. Ryan.