2010

FUNCTION AND REGULATION OF MATRIX METALLOPROTEINASE-1 IN GLIOBLASTOMA MULTIFORME

Monika Anand
Virginia Commonwealth University

School of Medicine
Virginia Commonwealth University

This is to certify that the dissertation prepared by Monika Anand entitled

FUNCTION AND REGULATION OF MATRIX METALLOPROTEINASE-1 IN GLIOBLASTOMA MULTIFORME has been approved by her committee as satisfactory completion of the thesis or dissertation requirement for the degree of Doctor of Philosophy

Helen L. Fillmore, Ph.D., Director of Dissertation

Sumitra Deb, Ph.D., School of Medicine

Joyce A. Lloyd, Ph.D., School of Medicine

Youngman Oh, Ph.D., School of Medicine

Zendra E. Zehner, Ph.D., School of Medicine

Sarah Spiegel, Chair, Department of Biochemistry and Molecular Biology

Jerome F. Strauss, III, M.D., Ph.D., Dean, School of Medicine

Dr. F. Douglas Boudinot, Dean of the School of Graduate Studies

JULY 29, 2010

FUNCTION AND REGULATION OF MATRIX METALLOPROTEINASE-1 IN GLIOBLASTOMA MULTIFORME

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at Virginia Commonwealth University

by

MONIKA ANAND

Master of Science

University of Calicut, Kerala, India

Director: HELEN L. FILLMORE, Ph.D.
ASSOCIATE PROFESSOR, DEPARTMENT OF NEUROSURGERY

Virginia Commonwealth University
Richmond, Virginia
August, 2010

Dedicated to

My parents

Sri Chaman Lal & Smt Lalita Anand

AND

My husband,

Prashanth Kotriki

Acknowledgments

Words cannot describe my deepest gratitude to Mom and Dad who have made innumerable sacrifices in giving me the best education and life. They have always encouraged me to fulfill my dreams with hard-work, dedication and single-minded focus. Their unconditional love, constant support and relentless prayers have helped tremendously in the completion of this work.

I express my sincere gratitude to Dr Helen L Fillmore for giving me an opportunity to work in her laboratory. I convey my heartfelt thanks to her for her excellent guidance and critical analysis of my work. She has been very supportive, encouraging and patient in channelizing my abilities towards becoming a scientist. Many thanks are extended to Dr William C Broaddus for giving valuable inputs and suggestions in the project. I will always cherish his enriching scientific discussions during our laboratory meetings. I admire Dr Timothy Van Meter for constantly taking out time from his busy schedule to discuss diligently my on-going experiments, results, analysis and troubleshooting. I deeply appreciate his willingness to help out with every issue arising out of our day-to-day experiments in the laboratory.

I am grateful to Dr Sumitra Deb, Dr Joyce A Lloyd, Dr Youngman Oh and Dr Zendra E Zehner for serving on my graduate committee. They extended support to me in every possible way by imparting their perspectives, opinions and critical comments in my project. Their guidance has helped me immensely in shaping and fulfilling this work.

Many thanks are extended to Dr Paul Dent for providing adenoviral vectors crucial for the second part of this study and Dr Adly Yacoub for his valuable guidance and review of data.

I am eternally indebted towards my best friend, soul-mate and critic, Prashanth. I would not be writing this thesis today without his presence in my life. He has been with me through the ups and downs of my stay at graduate school perpetually nourishing me with his classic light-heartedness and unwavering optimism. It has also been a wonderful experience discussing science with him. His intelligence, creativity, perseverance and sincerity in work have invariably given me inspiration to perform the best. I would also like to thank my brothers, Ranjan and Kapil and my dear friend, Anuradha for their love and encouragement that kept me motivated.

I extend many thanks to all my past and present colleagues, Dr. Justin McClain, Dr. Nicholas A Pullen, Dr. Anil Kumar, Archana Chidambaram and Thomas Gavigan for making the lab environment friendly and conducive to research. I would like to especially thank Dr Maria Redlak for helping me out by lending her lab supplies in times of need and listening to research ideas, joys and frustrations of my results.

I am extremely grateful to Mr Fenton N Hord and Mrs Patricia H Hord for providing financial support for this project. The Hord fund was established in VCU in memory of their son, Fenton N Hord, Jr. who became a victim of a brain tumor in 1991.

Table of Contents

List of Tables

List of Figures

List of abbreviations

AAA	Abdominal aortic aneurysm
ALA	5-aminolevulinic acid
ALL	Acute lymphocytic leukemia
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
APMA	Amino phenyl mercuric acid
BBB	Blood brain barrier
BSA	Bovine serum albumin
BCNU	1, 3-bis (2-chloroethyl)-a-nitrosourea
BMP	Bone morphogenic protein
BTSC	Brain tumor stem cell
CBTRUS	Central Brain Tumor Registry of United States
CCNU	N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea
CD44	Cluster differentiation 44
CDK4	Cyclin dependent kinase 4
CSF	Cerebrospinal fluid
CMV	Cytomegalovirus
CNS	Central Nervous System
CT	Computer tomography

CXCL12	C-X-C motif ligand 12
DC	Dendritic cells
DMEM	Dulbecco’s modified eagle medium
DN	Dominant Negative
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	experimental auto-immune encephalitis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme- linked immunosorbent assay
EPR	Epiregulin
ERK	Extracellular signal-regulated kinase
Ets-1	Ets Transcription Factor Protein-1
ETS	Erthroblastosis -26 family of transcription factors
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FLAIR	Fluid attenuated inversion recovery
G	Guanine nucleotide

GAGs	Glucosaminoglycans
GBM	Glioblastoma multiforme
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GPI	glycophosphitidyl inositol
Grb2	Growth factor receptor-bound protein 2
Gy	Gray
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IHC	Immunohistochemistry
IL	Interleukin
IR	Ionising radiation
JAK	Janus kinase
JNK	c-jun N-terminal kinase
kDa	kiloDalton
KPS	Karnofsky Performance Status
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase

MDM2	Murine double minute 2
MEK	MAPK activator
MEKK	MEK activator
MGMT	O6 methylguanine methyltransferase
MMP-1	Matrix-metalloproteinase-1
MMPI	Matrix metalloproteinase inhibitor
MT-MMPs	Membrane-type matrix metalloproteinases
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NF-1	Neurofibromatosis
NFκB	Nuclear Factor κ light chain enhancer of B cells
NRG	Neuroregulin
NSC	Neural stem cell
PAGE	Polyacrylamide gel electrophoresis
PAR-1	Protease-activated receptor-1
PBS	Phosphate buffered saline
PCV	procarbazine, carmustine and vincristine

PDE	Partial differential equation
PH domain	Pleckstrin homology domain
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphotidylinositol bisphosphate
PIP3	Phosphotidylinositol trisphosphate
PDGF	Platelet derived growth factor
PEA-3	Polyoma virus enhancer A binding protein 3
PET	Positron emission tomography
PKC	Protein kinase C
PMA	Phorbol myristic acid
PTCH-1	Patched-1
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma
RLU	Relative luciferase unit
RNA	Ribonucleic acid
RRE	Ras-responsive element
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase- polymerase chain reaction
Rsk	Ribosomal S6 protein kinase
SAPK	Stress activated protein kinase

SBE	STAT binding element
SH2 domain	Src- homology domain
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
SOS	Son-of-sevenless
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor-β
TIE	TGF- β inhibitory element
TIMP	Tissue inhibitor of metallproteinase
TMZ	Temozolomide
TNF	Tumor-necrosis factor
TP53	tumor suppressor p53
TPA	Tissue polypeptide antigen
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
WT	wild-type
WHO	World Health Organization

Abstract

REGULATION AND FUNCTION OF MATRIX METALLOPROTEINASE-1 IN GLIOBLASTOMA MULTIFORME

By Monika Anand, M.Sc

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

Virginia Commonwealth University, 2010

Major advisor: Dr Helen L Fillmore

Assocaite Professor, Department of Neurosurgery

Glioblastoma Multiforme (GBM) is an aggressive and fatal cancer of the brain. It is characterized with augmented morbidity and elusion to therapies due in part to the incessant infiltration and spread of tumor cells in normal brain. We investigated the function of Matrix metalloproteinase-1, an important enzyme noted to be responsible for invasion in other cancers, in GBM and its regulation by epidermal growth factor receptor

(EGFR) signaling. Previous studies from our laboratory demonstrated elevated levels of MMP-1 in GBM. Further studies indicated the involvement of MMP-1 in GBM invasion.

The GBM cell lines T98G, U251MG and U87MG were used for this study. In T98G cell lines, inhibition of MMP-1 by siRNA significantly suppressed basal *in vitro* invasion without impacting cell viability. The over-expression of MMP-1 was accomplished in U251MG and U87MG using the mammalian expression vector, pIRES, encoding full length MMP-1 cDNA. The MMP-1 over-expressing U251MG and U87MG cells exhibited significantly enhanced invasion *in vitro* with no modification in the cell proliferation rates.

A majority of GBM patients present defective EGFR signaling due to over-expression, amplification or mutation in the receptor. MMP-1 is known to be up-regulated by various stimulatory agents including growth factors. We examined the regulation of MMP-1 by EGFR activation and observed the induction of MMP-1 after EGF treatment. Inhibition of the receptor by pharmaceutic inhibitor treatment and genetic approaches led to reduction in MMP-1 levels. We also observed that this regulation is primarily mediated by the downstream MAPK pathway. Inhibition of MAPK and not PI3K pathway resulted in diminished MMP-1 protein levels even in the presence of EGF.

These studies demonstrate the importance of the EGFR-MAPK signaling pathway in the induction of MMP-1 in glioma cell lines. In addition, MMP-1 plays a role in glioma cell invasion *in vitro*. These results along with the reports of MMP-1 over-expression in GBM warrant future studies examining the function of MMP-1 *in vivo*.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Brain Tumors – Overview

Brain tissue is composed of two broad classes of cell types-neurons and glial cells. Although, neurons are considered to be the primary functional components of brain due to the generation and transmission of electrical impulses, a large portion of the brain is constituted by glial cells. These cells are of different types; astrocytes, oligodendrocytes, Schwann cells, ependymal cells and microglia. Glial cells provide physical support and nutrition to the neuronal cells (Jessen and Mirsky., 2005; Campbell and Gotz., 2002). A majority of primary brain tumors arise from glial cells (astrocytes, oligodendrocytes and ependymal) and are collectively regarded as glioma (Louis et al., 2007; Kleihues and Cavenee, 2000). Primary malignant CNS tumors account for approximately 2% of all cancers (Buckner et al., 2007). They are the leading cause of death in children and the third leading cause of cancer related death in adolescents and adults (15-34 years of age) (Jemal et al., 2009). The overall incidence rate of all primary brain and CNS tumors in the year 2004-2006 was 18.71 per 100,000 person-years with a propensity of slightly increased occurrence in females. The majority of brain tumors occur in the frontal, temporal, parietal and occipital lobes (CBTRUS 2009 Statistical report).

The occurrence of high grade malignant glioma is ascribable to certain genetic abnormalities and possibly environmental factors (Bello et al., 1994). Mutations in the type 1 neurofibromatosis gene (NF1), adenomatous polyposis

coli (APC) gene, Patched-1 (PTCH-1) gene and the tumor suppressor p53 (TP53) gene lead to genetic syndromes that pose a significant risk factor in the development of brain tumors. In addition to these germline mutations, high-dose ionizing radiation is a sole environmental factor that is proven to be conducive to formation of this neoplasm. Ionizing radiation treatment is given to patients suffering from acute lymphocytic leukemia (ALL) or non-Hodgkin lymphoma that predisposes them to increased risk of brain tumors especially gliomas (Buckner et al., 2007). Other environmental risk factors such as use of cellphones, head trauma, electromagnetic fields, N-nitroso compounds and occupational hazards have also been studied but with inconclusive results (Wrensch et al., 2002).

1.2 Classification

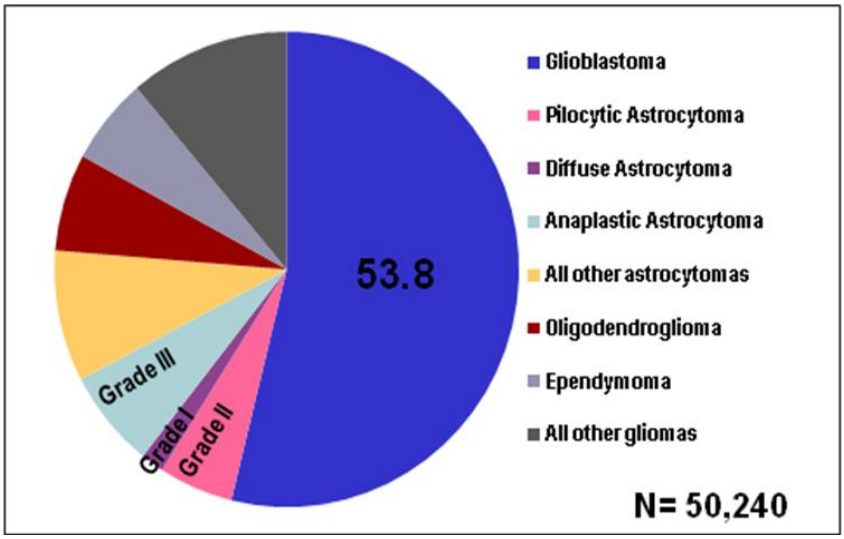
In the nineteenth century, prior to the beginning of the brain tumor classification system (1920s), all tumors originating in the brain were regarded as gliomas (reviewed by Ferguson et al., 2005). In 1867, Virchow stated that these tumors arise from interstitial brain tissue. In 1875, Golgi showed that some brain tumors are composed of star-shaped neuroglial cells. An extensive, thorough and organized work on the classification of brain tumors was started by Bailey and Cushing in 1922 (Bailey and Cushing, 1926). Thereafter, the World Health Organization (WHO) proposed a new comprehensive classification of neoplasms

affecting the CNS based on the cell-origin in 1993. As per the latest WHO classification (2007), gliomas belong to the neuroepithelial tumors of the CNS, arising from glial cells. Gliomas include astrocytoma, glioblastoma, oligodendroglioma, ependymoma and other neuroepithelial tumors. The incidence rate for neuroepithelial tumors is 1.4 times higher in males than in females (CBTRUS, 2009)

In addition to laying out a classification for brain tumors, WHO has also defined a grading system for gliomas that is based on certain histological markers; increased cellularity, cell proliferation, angiogenesis, necrosis and mitosis. The tumor grades increase with progressive cell proliferation and necrosis. Grade I is a low grade brain tumor, regarded as astrocytoma and it is well circumscribed, therefore considered relatively benign, affecting children and young adults. Eighty percent of patients with Grade I tumors survive greater than 20 years. Grade II is a low grade diffuse astrocytoma that grows slowly but has an inherent tendency to invade normal brain tissues and eventually progress to Grade III and IV. The median survival rate is highly variable; between five to ten years. Grade III (Anaplastic astrocytoma) demonstrates histological evidence of malignancy, including nuclear atypia and accelerated mitotic activity. Grade IV (Glioblastoma Multiforme, GBM) tumors are malignant, characterized with hyper-cellularity, profound vascular highly active at mitotic level, necrotic with extremely poor prognosis and fatal outcome (Louis et al., 2007). Overall, gliomas account for

33% of all tumors, of which glioblastomas are the most common, contributing 54% [**Figure 1.1**].

Figure 1.1 A major portion of primary brain and CNS tumors is comprised of non-malignant meningioma (~34%) followed by glioblastoma (~17%). Gliomas are tumors arising from glial cells and include astrocytoma, glioblastoma, oligodendroglioma, ependymoma, mixed glioma, malignant glioma NOS (not otherwise specified) and other rare types (CBTRUS, 2010). This figure shows the distribution of these various subtypes of gliomas (N=50,240). GBMs are predominantly represented in glioma (53.4%). The astrocytoma and oligodendroglioma together constitute 29% followed by small percentages of ependymomas and mixed gliomas. (CBTRUS Statistical Report, 2010, NPCR and SEER data from 2004-2006) NPCR= National Program of Cancer Registries. SEER=Surveillance, Epidemiology and End Results.


53.8
Grade III
Grade I
Grade II
Glioblastoma
Pilocytic Astrocytoma
Diffuse Astrocytoma
Anaplastic Astrocytoma
All other astrocytomas
Oligodendroglioma
Ependymoma
All other gliomas
N= 50,240

The incidence of glioma is higher in males (7.06 per 100,000 person years) as compared to females (4.96 per 100,000 person years).

1.3 Symptoms and Diagnosis

General symptoms associated with brain tumors may include headache, memory loss, cognitive impairment, seizures, visual, speech and sensory deficits, nausea and vomiting (Chang et al., 2005). Headaches tend to vary in intensity and are more frequent and severe in the morning. Seizures are presenting symptoms in patients with supratentorial tumors. Depending on the location of the tumor, the signs and symptoms vary. For example, in patients with tumors located in the parietal lobe, speech dysfunction and sensory loss are observed. Vision dysfunction may be the outcome of tumors in parietal or occipital lobe. Temporal lobe tumors are characterized by memory loss and behavioral changes. A tumor in the frontal lobe could lead to imbalance, speech deficits, seizures and/or dementia. Lack of muscle coordination is observed in patients with cerebellar tumors (Newton, 1994). Thus, a person presented with persistent headache with nausea, vomiting or seizures is normally evaluated for the presence of a tumor mass in brain.

Currently, a variety of imaging methods are being used to assess tumor mass, extent, localization and malignancy level. Computer tomography (CT) scan

and magnetic resonance imaging (MRI) technology reveal most of the anatomical information such as the size and location of the tumor. MRI also gives us information on mass effect, edema, necrosis and hemorrhage at high tissue contrast. Although, both CT and MRI images are of high spatial resolution, it is the MRI that delivers better resolution. MRI gives us a sequence of different sets of images of a brain tumor, each using a particular parameter- T1-, T2-, proton-, perfusion-weighted images. On a T2-weighted scan, water and fluid-containing tissue is bright and thus, edema can be seen on a T2-weighted scan as a bright region. An improvisation on T2-wighted scan is FLAIR (fluid attenuated inversion recovery) that separates water (dark) from edematous tissue (bright). Most brain tumors are hypotense on T1-weighted images and hypertense on T2-weighted, FLAIR images. In GBM, the blood brain barrier (BBB) is disrupted leading to the leakage of the contrasting media [such as gadolinium (Gd)] that can be seen as a contrast enhanced region. Thus, MRI with gadolinium is considered to be the better diagnostic tool for confirming a diagnosis (Jacobs et al., 2005).

There have been additions and improvisations in MRI technology that help a surgeon tremendously. Dynamic contrast-enhanced MRI assesses the vascular hyperpermeability of tumor vessels thereby helping a surgeon make predictions for tumor grade. Magnetic resonance spectroscopy (MRS) and positron emission tomography (PET) are other important tools that are being used to examine the molecular and metabolic tumor markers in heterogeneous tumors such as GBMs.

The imaging is followed by histopathological examination of tumor samples taken from the patient. Confirmation of tumor grade is made only after histological analysis of the tumor specimens is complete.

Glioblastoma Multiforme is the most aggressive and invasive tumor of all the CNS tumors and constitutes approximately half of the gliomas. The hallmarks of GBM that set them diagnostically apart from low grade gliomas are increase cellular proliferation, angiogenesis and pseudopallisading necrosis (Kleihues and Cavenee, 2000). Moreover, the invasive nature of this tumor renders surgical resection as a merely palliative treatment. Inevitably, the tumors recur due to the infiltration of tumor cells in a diffused manner to normal tissue. These recurring tumors are also highly resistant to therapies.

The average age of GBM diagnosis is approximately 55 years. The diagnosis is made using MRI scans (using gadolinium) of patients with GBM that show a ring-enhanced mass lesion with low signal intensity at the center. Thereafter, biopsy is conducted that confirms increased necrosis in the center and augmented vascularity in the ring-enhanced region. The surrounding region is composed of normal brain parenchyma that is being diffused with infiltrative tumor cells.

GBMs are categorized into either primary (*de novo*) or secondary glioma. In a general population, approximately 90% of the patients are presented with *de-novo* GBMs (Ohgaki and Kleihues, 2007). Primary glioblastomas are typically diagnosed at a terminal stage without any presence of a previous clinical or histopathological evidence of pre-existing less malignant lesion. The average age of patients with *de-novo* GBM is 62 years. Secondary glioblastomas develop gradually from a low grade diffused astrocytomas (Grade II) or Grade III anaplastic astrocytoma and are diagnosed in younger people (average age 45 years). These factors notwithstanding, the clinical outcome of a patient with GBM does not differ between primary or secondary GBMs. However, there are distinct molecular and genetic pathways in the development of primary and secondary GBM (Ohgaki and Kleihues, 2007). These pathways may have some impact on patient's outcome and response to therapy. Thus, studying these molecular events that lead to the genesis of GBM may help predict the outcome of a patient.

1.4 Treatment

1.4.1 Surgery

GBM poses greatest challenges to a physician due to its highly aggressive invasive pattern and resistance to therapies. Factors such as neurotoxicity after therapy, edema due to tumor capillary leakage resulting in intracranial hypertension and drug delivery complications due to blood-brain barrier make this

tumor highly difficult to treat. Surgery, radio- and/or chemotherapy are the mainstay treatment options in GBM. Grade I tumors are largely treated with surgical resection only whereas the treatment options in low grade astrocytoma vary from patient to patient. The clinical prognosis for Grade III and IV tumors is extremely poor, with Grade III anaplastic astrocytoma having median survival of two to three years and Grade IV GBM, a dismal ten to twelve months (Davis et al., 1999).

At the initial stages of treatment after GBM diagnosis, resection of the tumor by surgery is carried out based on the tumor location and its extent. However, the aggressive infiltration of the GBM tumor cells makes complete surgical resection of the tumor unachievable. In any case, surgical debulking of the tumor is vital for relieving intracranial pressure due to tumor mass and improving survival (Hentschel et al., 2003). Thus, surgery largely remains an important palliative procedure. It is important to note that complete resection is defined as the absence of contrast- enhancing tumor image seen on a postoperative MRI. Conclusions from a recent phase III control trial reveal that complete surgical resection could be obtained by performing fluorescence-guided resections with 5-aminolevulinic acid (ALA) – induced tumor fluorescence as compared to the conventional microsurgery (Stummer et al., 2006).

1.4.2 Radiotherapy

After surgical resection, the patients are analyzed for their general health. A conclusive plan and individual regimen of radio- and chemotherapy is decided upon. A better prognosis is obtained if a patient is young, has a high Karnofsky Performance Status (KPS), a high score on Folstein test and has promoter methylation of the O6 methylguanine methyltransferase (MGMT) gene. KPS is a scale that runs between zero (death) to 100 (disease-free state) and is based on parameters such as ability to take care of oneself, requirement of medical care, progression to disease etc. The Folstein test is a questionnaire that assesses a patient's performance in arithmetic, memory and orientation. MGMT is a repair enzyme that is involved in the GBM pathology. Methylation of the promoter region of MGMT inhibits the enzyme and improves prognosis in some patients (Krex et al., 2007; Esteller et al., 2000; Hegi et al., 2004). Radiation treatment has been consistently used as part of adjuvant therapy after it was established that it increased the median survival time from 4 months to 10 months (Walker et al., 1979). Varying doses, fractions and time interval between the fractional doses of radiation have been examined for best efficacy. The aggressive growth pattern in GBM along with the lack of sufficient blood supply makes the tumor environment largely hypoxic and necrotic. These conditions make the tumor highly resistant to radiation therapy (Mehta et al., 2004). To circumvent this issue, many radio-sensitizers have been tried, including misonidazole and metronidazole, without any positive results (Mehta et al., 2004).

Radiation treatment can be given externally (external beam radiation) or internally (brachytherapy). Brachytherapy involves placement of radioactive sources inside the body at or near the resection site. Patients with GBM are generally treated with external beam radiation; conventional and/or stereotactic. In conventional external beam radiation treatment, a single beam of radiation is delivered to the patient in multiple directions to target the tumor volume from all the sides. Radiation toxicity to nearby healthy tissues is a disadvantage to this procedure. Stereotactic radiation treatment involves delivering a high dose of radiation directly to the tumor/resected area in a single or two to three sessions. This type of radiation treatment minimizes the death of healthy tissue, requires lesser time, is equally or more effective but it is suitable to only small, well defined tumor masses (Buckner et al., 2007). The standard external beam radiation treatment consists of 25-35 daily treatments for over five to seven weeks for a total radiation dose of 60 Gys (Chandana et al., 2008). In patients above 70 years of age, a total of 50 Gys with 1.8 Gy fractions has been found to improve survival outcome (Keime-Guibert et al., 2007).

1.4.3 Chemotherapy

Studies in finding appropriate drugs for brain tumors have been ongoing for the past 30 years with very limited breakthroughs. Carmustine (BCNU) chemically 1, 3-bis (2-chloroethyl)-a-nitrosourea, was the first single drug used in

the treatment of malignant glioma in early 1970s (Barker et al., 1973; Wilson et al., 1976). In majority of patients, BCNU used to be given to patients intravenously but nausea and vomiting were experienced by patients within 2 hours of infusion. An improvised version of BCNU delivery method, Gliadel wafers, was developed (Wu et al., 1994). These small wafers coated with BCNU were placed directly to the site of tumor. However, this method too has its own complications. There was risk of toxicity to adjoining healthy tissues and decrease in blood count making the patient susceptible to infection and bleeding. Moreover, it was costly for most of the patients. Concurrently procarbazine was also developed and has been used in the clinical trials (Kumar et al., 1974). Over a period of time, due to dismal improvement in survival, multidrug treatments were initiated. The drug combination procarbazine, CCNU (lomustine) and vincristine (PCV) was found to be highly effective (Gutin et al., 1975). However, this therapy was accompanied with extremely severe side effects. Advent of Temozolomide (TMZ) (**Figure 2**) was a breakthrough in the chemotherapeutic treatment of GBM patients. TMZ (Temodar; Schering-Plough, Kelinworth, NJ) is an alkylating agent that induces methyl-groups at N-7 or O-6 positions of the guanine residues in DNA. Due to the damage to DNA, there is considerable cell death and tumor growth control. TMZ is very stable at acidic pH but its degradation increases between neutral to basic pH. The drug is administered in its pro-form and during physiological pH, it rapidly degrades and becomes activated.

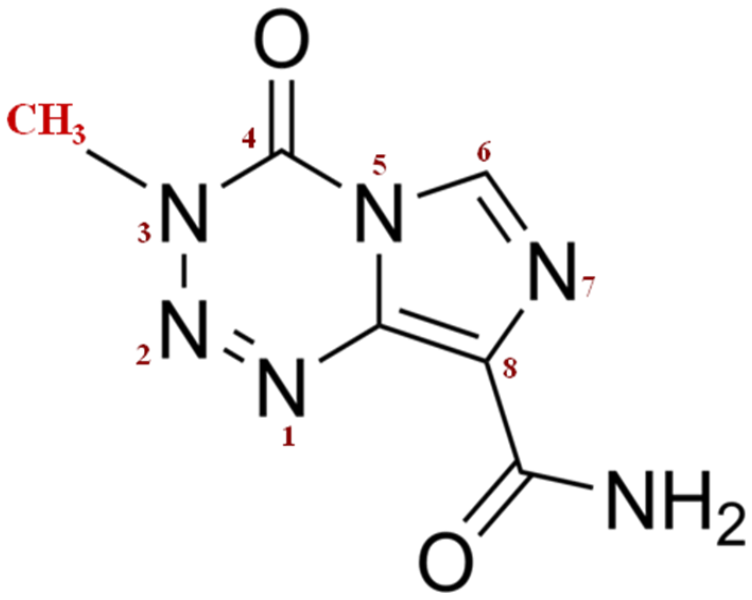


Figure 1.2. Chemical structure of Temozolomide

Upon hydrolysis in the physiological pH, the TMZ ring structure collapses releasing N_2 from (N at positions 2 and 3), CO_2 gas (C-4), 5-aminoimidazole-4-carboxamide (AIC) (N-1 and imidazole atoms), a natural component of urine, as by-products. The methyl group transfer takes place immediately after reaction with water/hydroxyl group.

Although there is considerable cell death due to TMZ, tumor cells may develop resistant mechanisms to it by expressing repair enzymes such as the above mentioned O6-methylguanine-DNA methyltransferase (MGMT) gene. TMZ induced methyguanine adducts are removed by MGMT as part of repair process. This MGMT-mediated resistance to TMZ is overcome by pre-treatment with O6-benzylguanine; a substrate analog for MGMT. TMZ itself is also used as a pretreatment to deplete the levels of MGMT in tumor cell population (Tolcher et al., 2003).

Clinical trials using TMZ have shown that it can be regarded as an oral single-agent drug superior to other drugs for recurrent GBM patients (Yung et al., 2000). Recently, the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) performed a randomized, multicenter, phase III trial to compare radiotherapy with TMZ as adjuvant followed by TMZ treatment for six months and radiotherapy alone. The patients with TMZ plus radiotherapy had a higher 2-year survival rate and increased progression-free survival as compared to the radiotherapy alone (Stupp et al., 2005). Such studies have made TMZ administration and fractionated radiotherapy following near complete surgical resection of tumor as the standard care in the treatment of GBM patients today.

Despite latest revolutionary surgical tools, newer and better methods of drug delivery, the disease is incurable and has very high morbidity rate. New insights into the glioma molecular biology may provide hope. As stated earlier, GBM is a heterogenous tumor with dysregulation of multiple signaling pathways due to genetic mutations. Using glioma biology as basis, new chemotherapies are underway. Most of these therapies target molecules that are involved in growth-factor mediated cell-signaling pathways that lead to increased cell proliferation and neo-angiogenesis.

1.5 Glioma Biology

As mentioned above, GBMs either develop de-novo, without any previous manifestation of the disease or they progress from low-grade astrocytomas. The important genetic alterations that lead to GBMs are listed in Table 1. GBMs are characterized by loss of heterozygosity (LOH) at chromosome 10q, epidermal growth factor receptor (EGFR) amplification, mutation or over-expression, $p16^{INK4a}$ deletion and loss or mutation in phosphatase and tensin homolog (PTEN). During development into secondary glioblastomas from low grade astrocytomas, the most characterized genetic alteration is mutation in TP53. This is followed by accumulation of other additional genetic mutations such as alterations in retinoblastoma protein (RB) gene, PDGF and PDGF-α over-expression, LOH at chromosome 19q and 10q and PTEN mutations. The primary

and secondary GBM also differ in the promoter methylation patterns and RNA expression profiles (Ohgaki and Kleihues, 2007). In addition, the DNA repair mechanisms that go awry in GBM include nucleotide excision, base excision and mismatch repair mechanisms. As mentioned earlier, MGMT is a mismatch-repair enzyme that helps in protecting a cell from alkylating agents generated by environmental damage. In addition, MGMT also imparts cancer cells a resistance mechanism to chemotherapeutic drugs that induce cell death by acting as DNA-alkylating agents. In a subset of GBM patients, MGMT gene is hyper-methylated. In these patients, treatment with DNA-alkylating drug has better prognosis (Nagarajan and Costello, 2009).

Genetic Events Responsible for GBMs
LOH 10q
EGFR amplification, mutation, over-expression
PTEN loss or mutation
p16INK4a deletion
TP53 mutation

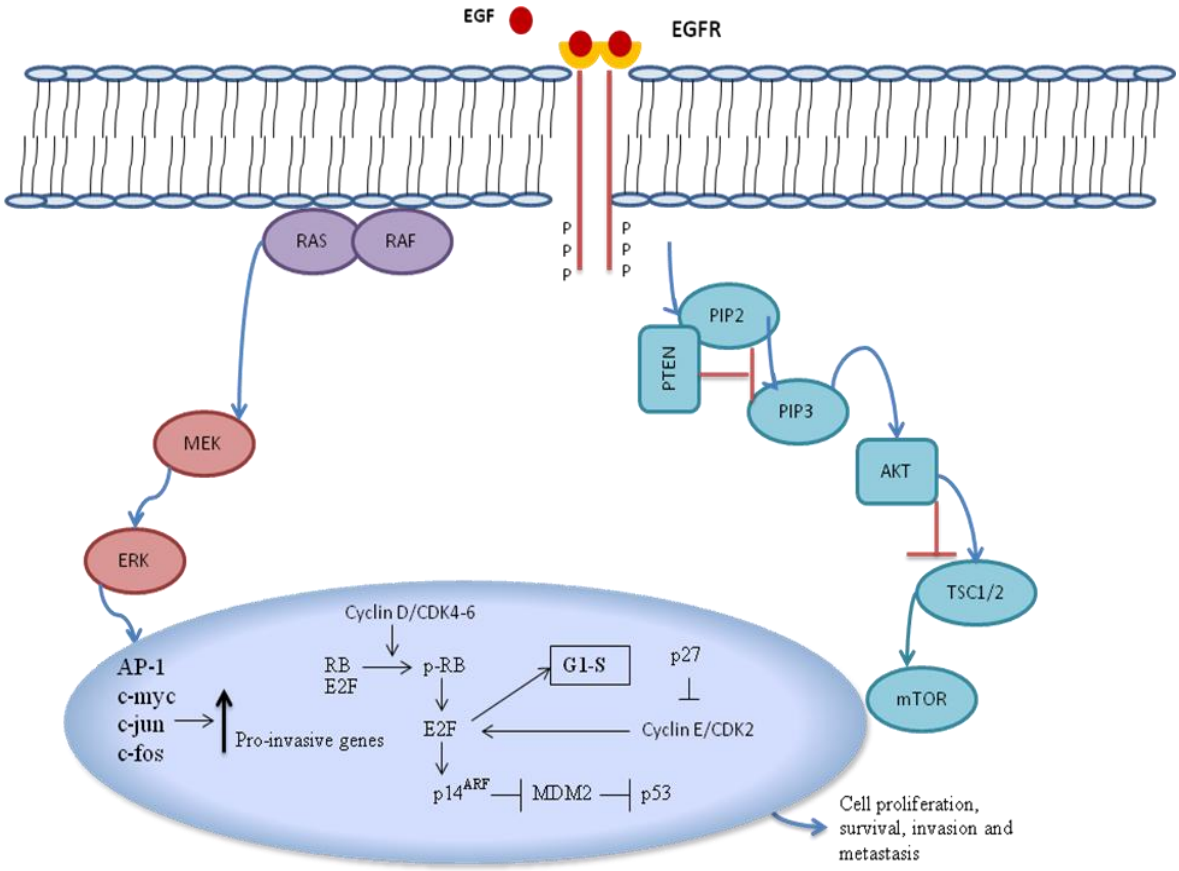
Table 1- Genetic Events in GBMs

Aberrant RTK (receptor tyrosine kinase) (EGFR, PDGFR, MET) signaling together with hyperactivated downstream pathways including RAS/MAPK, PI3K/AKT/mTOR, Protein kinase C (PKC) signaling, depletion of p53 and defects in RB pathway contribute significantly to glioma growth, proliferation, survival, vascularization and invasion in GBMs (Grzmil and Hemmings, 2010) (**Figure 3**). Amplification and over-expression of EGFR is more common in primary GBMs than secondary GBMs. EGFR amplicons are often mutated, with EGFRvIII mutation (deletion of exon two to seven), as the most prevalent form. The EGFRvIII variant is a constitutively active form of the receptor. This type of mutation has profound effects on cell proliferation, migration and vascularization. In addition, this truncated receptor also confers resistance to chemotherapeutic agent, cisplatin by modulation of BcL-xL and caspases in cell death pathways (Nagane et al., 1998).

In a vast majority of malignant brain tumors, including GBM, mutations in the TP53 pathway and/or retinoblastoma (RB1) pathway are also prevalent. TP53 mutations are the hallmark event in secondary GBMs. They also occur in primary form of GBM, although at a lower frequency (<30% of the cases, Ohgaki et al., 2004). Mutation pattern involving G: C → A: T, frequently at two hotspot codons 248 and 273 in TP53 gene, are directly associated with progression to secondary GBMs. In GBMs, where TP53 mutation is absent, there is an amplification of MDM2 (murine double minute) that rapidly degrades TP53 protein (Biernat et al.,

1997). TP53 protein gets activated after DNA damage in response to radiation or chemical mutagens. MDM2 binds to TP53 facilitating its degradation by ubiquitinization. Conversely, MDM2 gene is up-regulated by TP53. In normal cells, this regulatory feedback loop is maintained but in a transformed cell, this regulation is dysfunctional. Another gene product, $p14^{ARF}$ binds to MDM2 and prevents MDM2 –mediated degradation of TP53. Thus, loss of TP53 function, in a tumor cell, may result from aberrant expression in MDM2 and $p14^{ARF}$ gene.

Figure 1.3 Major pathways in gliomagenesis Schematic representation of EGFR signaling in glioma. Epidermal growth factor binds to its receptor EGFR leading to receptor dimerization and autophosphorylation at various tyrosine (Y) residues. This further results in activation of several downstream signaling pathways among them ras-raf-MAPK, PI3K/AKT and JAK/STAT are prominent. MAPK activation affects DNA synthesis and cell proliferation and PI3K/AKT has role in cell survival.


EGF
EGFR
RAS
RAF
P
P
P
P
P
P
PIP2
PTEN
PIP3
AKT
TSC1/2
mTOR
MEK
ERK
Cyclin D/CDK4-6
RB
E2F
p-RB
G1-S
p27
AP-1
c-myc
c-jun
c-fos
Pro-invasive genes
E2F
Cyclin E/CDK2
p14ARF
MDM2
p53
Cell proliferation,
survival, invasion and
metastasis

RB1 (retinoblastoma 1) pathway is equally dysregulated in both primary and secondary GBMs. RB1 protein is phosphorylated by the CDK4/cyclin D1 complex releasing E2F transcription factor which induces genes that aid in $G_1 \rightarrow S$ transition in the cell cycle. Another important protein, $p16^{INK4a}$ binds to CDK4 and inhibits RB1 phophorylation, thus inhibiting the $G_1 \rightarrow S$ transition.

Loss of heterozygosity (LOH) at chromosome 10q is a major genetic alteration that occurs in 60-80% population with GBMs (Fujisawa et al., 2000, Rasheed et al., 1995). As an exclusive event, complete loss of chromosome 10 may be seen only in primary GBMs. The commonly deleted loci at chromosome 10 are 10p14-p15, 10q23-24 (PTEN) and 10q25-qter that encodes several tumor suppressor genes.

Promoter methylations of the $p14^{ARF}$, $p16^{INK4a}$, RB1, MGMT and tissue inhibitor of metalloproteinase-3 (TIMP-3) genes are common events in secondary GBMs (Nagarajan and Costello, 2009). In a cDNA expression profile study conducted to assess differences in gene expression, IGFBP2 gene was significantly over-expressed in primary GBM (Godard et al., 2003). Microarray analysis with subsequent gene annotation revealed alterations in genes involved in cell cycle regulatory pathways in secondary GBM and loss of function in genes involved in signaling pathways in primary GBMs (Tso et al., 2006). EGFR, MDM2 and MMP-9 genes were elevated in primary GBM as observed from both

arrays and immunohistochemistry profiles (Watanabe et al., 1996; Tso et al., 2006; Biernat et al., 1997).

1.5.1 Glioma Biology as basis for developing novel drug targets

GBM treatment remains unfruitful due to numerous factors that include lack of effectiveness and high cytotoxicity of the drugs, aggressiveness of the disease and inability for complete surgical resection of the tumor. These pitfalls in the therapy have led to the development of novel drugs that target cellular signaling pathways in gliomagenesis. GBM is a heterogeneous tumor wherein multiple signaling pathways are dysregulated. Targeting different molecules simultaneously is important for a successful outcome. In tumor cells, several diverse signaling pathways are constitutively active. Some of these pathways converge to common downstream transcriptional factors. Developing a drug against a target upstream in a signaling pathway would not be as beneficial as developing multiple downstream targets because inhibition of upstream molecules in a signaling pathway may result in other compensatory mechanisms leading to drug resistance.

Some of the small molecule inhibitors that seem promising are inhibitors that target EGFR (erlotinib), Akt (perifosine), farnesyl transferase (lonafarnib), Met (XL184), mTOR (sirolimus), PI3K (BEZ235), PDGFR (imatinib) and TGF-β

(AP12009). A complete comprehensive list of inhibitors to such oncogenic molecules is given in **Table 2** (Adamson et al., 2009). The anti-angiogenesis approach has been significant with regards to GBM because aggressive growth of the tumor leads to hypoxic conditions that in turn cause neovascularization. Recently the developed anti-angiogenic drug, Bevacizumab, a monoclonal antibody against vascular epidermal growth factor (VEGF) has been FDA approved in 2008 for use in breast cancer patients. This drug is a strong candidate for GBM patients and is under clinical trials for GBM therapy (Adamson et al., 2009). Other anti-angiogenic drugs under clinical trials for GBM are the ones that target $\alpha_v\beta_5$ integrins (cilengitide), hepatocyte growth factor (HGF) (AMG-102) and VEGFR (sunitinib) (Adamson et al., 2009).

Table 2 Current active clinical trials for GBM in United States (Adamson et al., 2009, www.clinicaltrials.gov). The most common chemotherapies under investigation include small-molecule targeted therapies (directed against molecules in signaling pathways), anti-angiogenic therapies, immunotherapies and gene therapies. The drugs listed here are primarily against the molecues aberrantly activated or over-expressed.

Drug name	Target molecule
Perifosine	AKT
Erlonitib, Gefinitib	EGFR
XL 184	Met
Prinomastat	MMP
Dasatinib, Imatinib	PDGFR
Rapamycin, serolimus	mTOR
BEZ 235, XL 765	PI3-kinase
Sorafenib	Raf
TLN-4601	Ras
PTK 787, semaxanib	VEGFR
AP 12009	TGF-β
AT 13387	HSP90

Immunotherapy

In addition to above therapies, another promising approach is using immunotherapy where patients own immune system is used to target the tumor cells (Kanaly et al., 2010). Injections of tumor peptides by RNA, DNA, Viral particles or whole cells leads to sensitization of these lymphocytes against a tumor antigen leading to specific clearance of tumor cells without affecting the normal healthy tissue. For example, TP-38 is a genetically engineered protein that has the EGFR ligand (TGF-α) fused with *pseudomonas* exotoxin. The N-terminus of TGF-α protein binds with EGFR followed by TP-38 exotoxin cleavage and cell death (Rainov and Soling, 2006).

In the passive approach of immunotherapy, the lymphocytes are removed from a patient's body, expanding those cells that demonstrate activity against the tumor cells and reintroducing them back in the patient. For example, dendritic cells (DCs) are loaded with tumor specific peptides (EGFRvIII), tumor lysates, tumor stem cell mRNA and injected into the GBM patient as vaccines (Parney et al., 2000). In one such Phase I clinical trial of DC immunotherapy, survival was prolonged to 133 weeks as compared to 33 weeks in control patients and the therapy was found to be completely safe (Yu et al., 2006). In a more tumor-specific target approach, EGFR vIII vaccine has been developed and has delivered promising results in two separate clinical trials (Schmittling et al., 2008; Parney et al., 2010).

Brain Tumor Stem Cells

During developmental stages, mammalian brain contains a population of neural stem cells (NSCs) that have the capacity to self-renew and proliferate. The presence of such NSCs in adult human brain has been reported in separate studies (reviewed by McKay 1997; Uchida et al., 2000). These NSCs gained great significance in CNS repair mechanisms post-injury (Laywell and Steindler, 2002). Presence of neural progenitor cells in adult brain strongly links normal physiological development of brain with brain cancer (Pardal et al., 2003).

Recently, brain tumor stem cells (BTSCs) have been identified and isolated from brain tumor specimens from patients by using a neural stem cell marker CD133 (Ignatova et al., 2002; Singh et al., 2003, 2004). These $CD133^+$ BTSCs show high capacity to self-renew and proliferation in-vitro and in-vivo. The $CD133^+$ tumor neurospheres express stem-cell marker, nestin during neural stem cell culture conditions and upon changing culture conditions to serum-containing media, they differentiate into a mixed population of neurons, astrocytes and oligodendrocytes. More recently, it has been reported that isolation of $CD133^-$ cells from GBM also form orthotopic tumors in mice and give rise to $CD133^+$ cells in vivo (Wang et al., 2008). This suggests that there may be other tumor initiating cells in GBM that do not express CD133.

Understanding the biology and gene expression profile of BTSCs would provide means to test novel therapeutics strategies. Prominent signaling pathways that are vital for survival of BTSCs are PI3K, NOTCH, Wnt, SHH. These pathways are needed to be targeted for elimination of BTSCs from tumors. Another approach would be to promote differentiation in BTSCs as it has been found that using bone-morphogenic proteins (BMPs) induced differentiation and prolongs survival in mice models (Atkinson et al., 2009, Hadjipanayis et al., 2009).

1.6 Invasion

1.6.1 General overview of Invasion and metastasis in cancers other than GBM

In any cancer setting, tumor cell invasion and metastasis dramatically increase the morbidity and mortality due to cancer. To achieve invasive properties, a subpopulation of tumor cells must recognize the ECM molecules present in the vicinity of the cell, attach to these molecules by specific receptors present on the cells and modify the ECM by cleaving physical barriers paving a way for the tumor cell to migrate to other parts of the body (Geho et al., 2005).

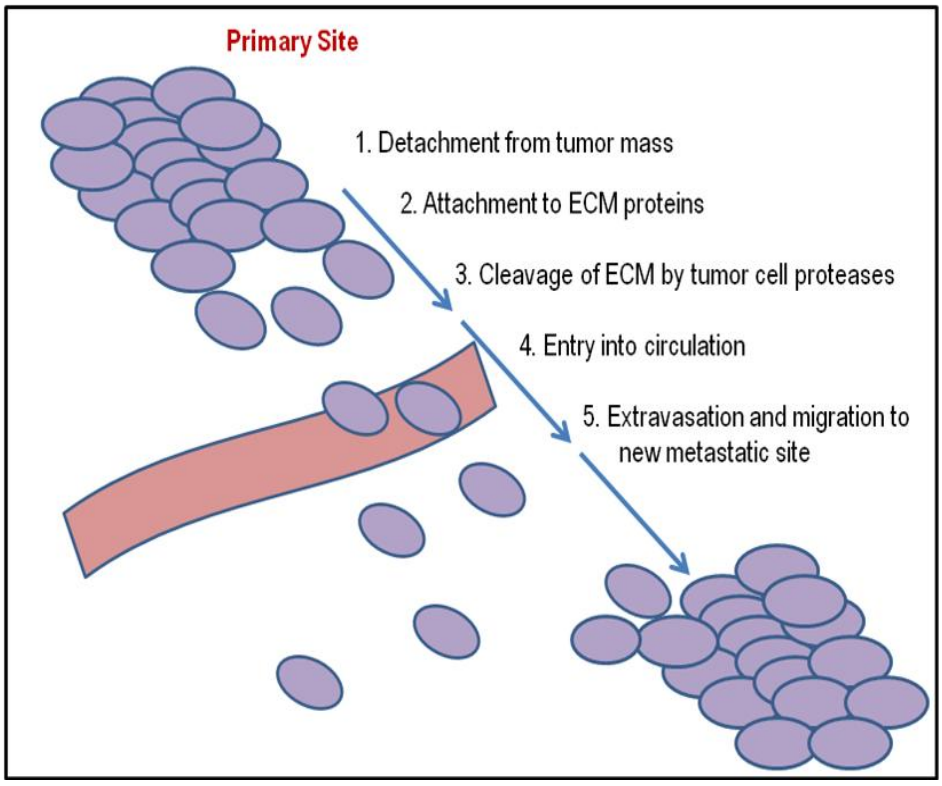
Figure 1.4: **Invasion/Metastasis in cancer**

Invasion process has three basic steps:

a) The tumor cells detach from the solid tumors at the site of origin.

b) The cells then adhere to the extra-cellular matrix (ECM) proteins by expressing specific receptors that bind to ECM proteins.

c) The cells release proteases and other molecules that aid in the degradation of complex ECM molecules thereby paving a way for the tumor cell to navigate through adjoining normal tissue.


Primary Site
1. Detachment from tumor mass
2. Attachment to ECM proteins
3. Cleavage of ECM by tumor cell proteases
4. Entry into circulation
5. Extravasation and migration to new metastatic site

Following invasion through ECM and blood vessels, the tumor cells migrate and attach to a new location. The new tumor mass is formed by up-regulating complex molecular machinery that aids in the process of proliferation, growth and neo-angiogenesis. These molecular mechanisms are similar to the ones that aid in normal physiological growth. During normal development, the cellular migration is tightly controlled by organism's homeostasis. In contrast, during metastasis, there is an unchecked constitutive up-regulation of molecular mechanisms that eventually leads to the death of the organism.

At the initial phase of the invasion process, interaction between tumor with ECM and stromal cells (Liotta et al., 2001) are mediated by cell-cell, cell-ECM contact and secretion of soluble factors by tumor cells that interact with ECM and stromal cells. The cell-cell and cell-ECM contacts are made by special class of adhesion receptor family of proteins that include selectins, immunoglobin cell-adhesion molecules, cadherins and integrins. The most widely studied cell adhesion receptor protein is integrin family of receptors. Integrins are heterodimeric transmembrane proteins that bind to specific ECM molecules at the extracellular side and specific cytoskeletal (CSK) adaptor proteins intracellularly (Giancotti and Ruoslahti, 1999) such as focal adhesion kinase (FAK) (Guo and Giancotti, 2004). In normal physiology, when a cell is detached from the ECM, it does not survive, a process referred to as "anoikis" (Frisch and Ruoslahti, 1997). Tumor cells escape anoikis by activating PI3-kinase/AKT pathway that inhibits

caspases (Frisch and Ruoslahti, 1997). Ligand binding to integrins leads to integrin-clustering and recruitment of intracellular signaling components following which Rho family of GTPases, MAP and PI3-kinases activate. Receptor tyrosine kinases, integrins and G-protein coupled receptors co-ordinate and get activated during tumor cell's transformation to a migratory and invasive phenotype. Cytoskeletal changes like actin-polymerization and myosin-contraction are mediated by Rho-kinases and cause polarization of the cell, membrane extension and cell substratum attachment in the migration process.

Tissue remodeling by matrix degradation and turnover are important events during physiological development. These events are also an integral part of wound healing, tumor necrosis and inflammation. The most important enzymes that are at the forefront of matrix degradation are matrix metalloproteinases (MMPs). Due to the proteolytic activity of MMPs, the rigid barriers of ECM are removed, latent forms of growth factors, proteases and chemotactic agents are activated and bioactive molecule within ECM are generated. In addition, the integrin binding sites in the ECM become exposed to interact with cells for transmitting migratory and survival signals (Davis, 1992).

1.6.2 Invasion in Glioblastoma Multiforme (GBM)

One of the important underlying reasons for the failure of GBM therapies is the ability of GBM cells to invade local brain parenchyma. Maximal surgical resection only slightly helps in improving patient's outcome as by the time the disease is diagnosed; tumor cells may have already infiltrated and cannot be identified. Thus, there is a dire need for therapeutic approaches that target the invasion process in GBM (Davis and McCarthy, 2001).

Brain tumor cells follow the basic steps of invasion with the unique exception that they rarely invade outside the brain tissue and metastasis by GBM cells is confined to within the brain tissue. There is no invasion and extravasion in and through the vasculature (Burger and Scheithauer, 1994; Hunter et al, 2003; Kleihues and Cavenee, 2000). The invasion pattern followed by primary brain tumors is different from the tumors that metastasize to the brain from other parts of the body. Primary brain tumors invade aggressively to wider margins within the brain, generally as single cells, some have the tendency to invade along the periphery of blood vessel walls or along white matter tracts in Virchow-Robins space (Fillmore et al., 1999; Bellail et al., 2004). These findings suggest that the migration of invading cells is dependent on specific substrates present in the microenvironment of CNS. Metastatic tumors that invade to brain generally have a well-defined mass and invade as groups of cells. Thus, a metastatic brain tumor

has relatively better response to surgical resection as compared to a primary malignant brain tumor.

Various studies have been conducted to study the migration pattern of GBM cells (Swanson et al., 2000, 2003; Mandonnet et al., 2003; Harpold et al., 2007). These studies are based on *in-vitro* experimental models (tumor spheroids) of tumor invasion and suggest that various patterns of migrations are followed by GBM cells including isolated islands, branching and dispersion both near and away from the tumor boundary. These results have been corroborated by a mathematical model based on partial differential equations (PDEs) that use glioma cell density and concentration of nutrients, proteases such as MMPs and ECM (Kim et al., 2009). It was shown that different dispersion patterns arise from the differences in haptotactic, chemotactic and cell-cell adhesion factors. The chemotactic flux is generated when tumor cells get attracted to glucose for energy requirement (Warburg effect) and move in the direction of glucose gradient. The model was based on the consideration that brain ECM is a deformable mass of fibers that is constitutively being degraded and remodeled by MMPs. Using this model, they observed that increasing cell-cell adhesion forces slowed migration confirming earlier reports of requirement of loss of cell-cell contacts for invasion in T98G cells (Perego et al.,2002).

ECM in brain mainly consists of hyaluronan, proteoglycans, tenascin-C and thrombospondin. Due to low abundance of fibrillar collagen, laminin and other molecules that impart strength to the ECM, it is largely amorphous and loosely bound and therefore difficult to study (Chintala et al., 1996). Proteoglycans such as chondroitin, heparin sulphates and glucosaminoglycans (GAGs) are present in brain parenchyma (Chintala et al., 1996). Other proteoglycans present in the brain are hyaluronic acid (HA), tenascin and cytotactin (**Table 3**).

Glias limitans externa	Collagen (I, III, IV) Laminin Fibronectin Heparan sulfate
Vascular basement membrane	Collagen (IV, V) Fibronectin Laminin Vitronectin Heparan sulfate
Brain parenchyma	Hyaluronic acid Dermatan sulfate Chondroitin sulfate Hyaluronectin Tenascin

Table 3: Components of ECM in brain (Adapted from Chintala et al., 1996)

The components of ECM continuously change during development and diseased states modulating brain cell growth, differentiation and invasion by different mechanisms. Hyaluronic acid (HA) is increased several fold in primary brain tumors comparable to the levels present during developmental stages of the brain. HA has been regarded to be involved in facilitating the tumor migration and invasion using specific cell surface receptors, cluster differentiation 44 (CD44) (Akiyama et al., 2001). Tenascin C is also up-regulated in brain tumors and its levels correlate with tumor grade (Higuchi et al., 1993; Zagzag et al., 1995). The expression of type IV collagen and laminin has also been reported to be found in the vessel walls along the endothelial glomerulus-like proliferations (Bellon et al., 1985). Type V collagen and fibronectin is present in vessel walls in the basement membrane and perivascular matrix and type 1 and III collagens are observed in the vessel walls and perivascular connective sheet (Bellon et al., 1985).

1.7 Matrix Metalloproteinases (MMPs)

1.7.1 Nomenclature and Classification

MMPs are a large family of zinc-dependent endopeptidases that collectively have the ability to cleave every component of ECM. The first member of the family, MMP-1, also referred to as collagenase-1, was described by Gross and Lapiere in 1962 while studying tadpole morphogenesis (Gross and Lapiere, 1962). Since then, 23 members of the MMP family have been found to

be expressed in human. MMPs play a crucial role in normal physiological processes like tissue remodeling during embryonic development (Page-McCaw et al., 2007), wound healing (Parks et al., 2004) and cancer (Egeblad and Werb, 2002). Historically MMPs have been divided into four categories depending on their substrate-specificites: Collagenases, Gelatinases, Stromelysins and membrane-type MMPs (MT-MMPs) (**Table-4**). Collagenases act on collagens type I, II, III, fibrillar collagens type I, II, III, VI, IX and include MMP-1, -8 and -13. MMP-18 is also a collagenase found in *Xenopus* species but it is not expressed in humans. There are two groups within gelatinases- Gelatinase A (MMP-2) and Gelatinase-B (MMP-9). Their primary substrate is gelatin type I, II, III but they can also act on collagen type IV, V, VII, X, fibronectin and elastin. Stromelysins are also divided further into three subgroups- stromelysin 1, 2 and 3. They act on fibronectin, laminin, collagens type II, IV, V, IX, gelatin type I, III, IV, V. Stromyelin 3 (MMP-11) is a unique enzyme that does not cleave ECM molecules such as collagen, laminin, elastin and fibronectin. Most of the membrane-type MMPs cleave progelatinase-A. These membrane-tethered MMPs are important for regulating cell behavior as they influence cell-surface and ECM activity. The secreted MMPs also aid in the signaling mechanisms by cleaving latent growth factor molecules in the ECM. In addition, they can also bind to the integrins or CD-44 cell surface receptors. Each member protein is encoded by a different gene and most of the genes for MMPs are clustered on chromosome 11 indicating a common evolutionary origin. The region Ch11q23, where MMP

genes are localized, is found to be amplified in several solid tumors (Rooney et al., 1999). The MMPs now are categorized on the basis of their structure (Egeblad and Werb, 2002)

Category	Name	Substrate
Collagenases	MMP-1, -8, -13	Collagens type I, II, III
		Fibrillar collagen I, II, III, VI, IX
Gelatinases	MMP-2, -9	Gelatin type I, II, III
		Collagen type IV, V, VII, X
		Fibronectin, Elastin
Stromelysins	MMP-3, -10, -11	Collagen type III, IV, V, IX
		Gelatin type I, III, IV, V
		Fibronectin, Laminin, Casein
Membrane-type MMPs	MMP-14, -15, -16, -17	progelatinase-A (MMP-2)

Table 4: Nomenclature and Substrate specificities of MMPs

(Adapted from Egeblad and Werb, 2002)

1.7.2 Structure

Structurally, MMPs have distinct domains with some highly conserved regions. The MMPs have been divided into eight distinct structural groups **(Figure 4).** These groups have a common basic structure called as the "minimal domain". The minimal domain consists of an amino terminal signal sequence (Pre) which is cleaved by the signal peptidase for facilitating the enzyme's entry into endoplasmic reticulum (ER). The Pre-domain is followed by a "Pro" domain consisting of a thiol group (-SH) and a furin- cleavage site. The pro-domain connects to a catalytic domain that has a Zn^{2+} atom binding site. The cysteine sulfhydryl group binds covalently to the Zn atom in the catalytic domain. This keeps the enzyme in its latent state. To activate the enzyme, the cystein to Zn bond has to be disrupted by either normal proteolytic removal of pro-domain by serine proteases or by ectopic disruption of cystein-Zn interaction (cystein switch) (Sternlicht and Werb 2001). Intracellular furin-like proteases that target the furin-recognition motifs (Fu) in the catalytic domains can cleave the prodomain and activate the enzyme intracellularly. The covalent bond can also be disrupted by chemicals such as amino-phenyl mercuric acid (APMA) or other serine proteases present in the ECM such as plasminogen activators (PA). In addition to this minimal domain, simple hemopexin domain containing MMPs have at the C-terminus a hemopexin-like domain that is connected to the catalytic domain by a hinge (H) region. There is a di-sulfide bond (S-S) between the first and the last subdomain of hemopexin domain. This hemopexin-like domain mediates the

interaction of the enzyme with other proteins such as the tissue inhibitors of metalloproteinases (TIMPs), cell surface molecules and proteolytic substrates. The gelatin binding MMPs have collagen-binding type II motifs of fibronectin (Fi) present in the catalytic domain. The furin-activated secretory MMPs have furin-proteases recognition motifs in their catalytic domain that cause intracellular activation of these MMPs. Membrane anchored MMPs include MT-MMPs that have a transmembrane domain at the C-terminus of the enzyme with a small cytoplasmic domain and glycosylphophatidylinositol (GPI) anchor or an amino-terminal signal anchor (SA). The MMP-23 (Type II transmembrane MMP) has a unique cystein array (CA) and immunoglobulin (Ig)-like domains (Egeblad and Werb, 2002).

Figure 4 Schematic of different structural categories of MMPs

The MMPs have been divided into eight categories based on their structure. All MMPs except type II MT-MMPs have a pre domain followed by a pro domain consisting of a thiol (SH) group. Type II MT-MMPs have signal anchor (SA) at the N-terminus. The catalytic domain has Zn atom binding site (Zn) or cystein array (CA). Some MMPs have collagen binding type II motifs of fibronectin (Fi) in the catalytic domain. The pro-domain may also include furin-proteases recognition motif (Fu) and Vitronectin (V). The hemopexin domain is connected to catalytic domain by a hinge region (H). The C-terminus of some MMPs may have glycosylphophatidylinositol (GPI) anchor or immunoglobulin (Ig)-like domains. (Adapted from Egeblad and Werb, 2002)

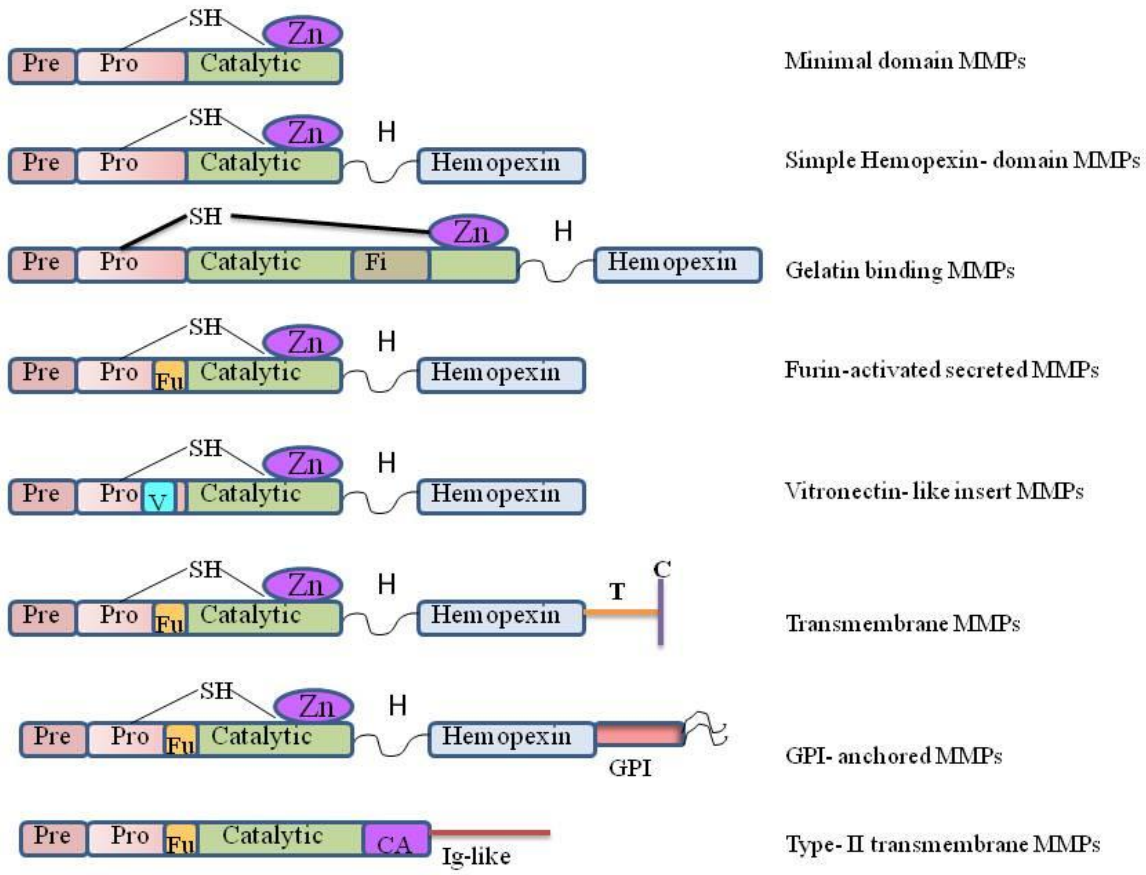

SH
Zn
Pre
Pro
Catalytic
Minimal domain MMPs
H
Hemopexin
Simple Hemopexin- domain MMPs
Fi
Gelatin binding MMPs
Fu
Furin-activated secreted MMPs
V
Vitronectin- like insert MMPs
T
C
Transmembrane MMPs
GPI
GPI- anchored MMPs
CA
Ig-like
Type- II transmembrane MMPs

Figure 4

1.7.3 Functions of MMPs

The function of MMPs in normal physiological conditions is controlled and regulated at various levels depending upon the requirement. A balance is maintained between expression of MMPs and their proteolytic degradation and inhibition by endogenous inhibitors. ECM is a biologically active and dynamic tissue that is continuously being remodeled and harbors various growth factors and signaling molecules. Any changes brought about in ECM affect cell cytoskeletal, adhesion machinery, growth and differentiation. Thus, by degrading ECM, MMPs are indirectly influencing these important events that shape cell behavior.

Functions of MMPs in normal physiology

Although MMPs are traditionally known to have ECM degradation as their primary function, recent work in MMP biology has shown that these enzymes can perform their proteolytic function to conduct many important physiological processes (Page-McCaw et al., 2007). MMPs can alter the epithelial tissue architecture by cleaving cell-cell junctions and cleave ECM molecules to clear the path for cell movement. They can also generate signaling molecules from ECM that may act by autocrine or paracrine mechanisms. For example, cleavage of collagen IV α3 chain by MMP-9 yields a new bioactive molecule, tumstatin, an anti-angiogenic peptide that binds to integrin receptor (Hamano et al., 2003). MMPs can also cleave latent bioactive molecules altering

cell behavior. For example, cleavage of vascular endothelial growth factor (VEGF) modifies its structure and affects its binding affinity to the receptor (Lee et al., 2005). MMPs can deactivate a signaling molecule by cleaving it thereby affecting cellular proliferation and motility. For example, MMP-2 cleaves CXC-motif ligand-12 (CXCL12) and inactivates it thereby making the host more susceptible to HIV-infection (McQuibban et al., 2001). Cleavage of collagen type XVIII results in a fragment, endostatin, which is an anti-angiogenic molecule. MMP-1 and MMP-3 cleave insulin-like growth factor binding protein-3 (IGFBP-3) present in ECM releasing IGF bound to it (Fowlkes et al., 1994). MMP-1 and MMP-3 also degrade proteoglycan perlecan present in ECM to release FGF (Whitelock et al., 1996). MMP-1 mediated type I collagen degradation is necessary for epithelial cell migration and would healing in culture models (Pilcher et al., 1997).

MMPs also perturb membrane signaling by cleaving growth factors, tyrosine kinase receptors, cell-adhesion molecules, cytokines, chemokines and other MMPs. Cleavage of membrane proteins such as E-cadherin and CD44 by MMPs results in an increase in cell migration and invasion (Noe et al., 2001; Kajita et al., 2001). Other examples of membrane protein shedding are cleavage of syndecan by MT1-MMPs resulting in an increased cell migration (Endo et al., 2003). TGF-β is released from its latent state by membrane localized MMP-9 (Yu and Stamenkovic, 2000).

MMPs in bone remodeling

MMPs are important in bone development and remodeling. MMP-9 knockout mice have defects in endochondral ossification, an important step in bone development (Vu et al., 1998). Although MMP-9 is also found to be highly over-expressed during bone healing after a fracture, it is not mandatory for healing process (Colnot et al., 2003). MMP-13 is required for bone remodeling and the transition from cartilage to bone at the growth plates of long bones (Stickens et al., 2004; Inada et al., 2004). MMP-14 knockout mice are lethal with gross defects in connective tissue remodeling (Holmbeck K et al., 1999; Zhou Z et al., 2000). Loss of function mutations in MMPs lead to defects in bone developmental diseases. MMP-2 mutation causes a rare osteolytic syndrome (Martignetti et al., 2001). Mutations in MMP-20 results in tooth enamel defect amelogenesis imperfect (Kim et al., 2005). Mammary gland development during puberty requires MMPs mediated degradation of basement membrane and ECM for restructuring vascular network (Sternlicht et al., 2006).

MMPs and Vascular development MMPs mutants do not show any defect during embryonic vascular development but postnatal vascular remodeling and angiogenesis is severely affected. MMP-9 mutants show defects in angiogenesis at the growth plate of long bones (Vu et al., 1998). MMP-2 mutant mice show reduced vascular growth (Kato et al., 2001) but MMP-14 mutant mice lack it altogether (Zhou et al., 2000). Such defects in vasculature could be

attributed to important functions of MMPs such as proteolysis of type I Collagen, modification of PDGF and VEGF.

In addition to developmental events, MMPs also contribute in maintaining cellular balance during inflammation and wound healing (Parks et al., 2004). MMP-7 mutant mice are more susceptible to bacterial infections due to the inability of MMP-7 mutant mice to proteolytically cleave and activate endogenous antibiotic, pro-cryptdin (Wilson et al., 1999). MMP-7 mutant mice do not show closure of wounds due to absence of MMP-7 mediated cleavage of E-cadherins (McGuire et al. 2003). Injury and infection produce inflammation by inducing leukocyte migration towards chemoattractants. The formation of chemoattractants requires MMP- mediated cleavage of syndecan-1 ectodomain. It has been found that neutrophil migration is defective in MMP-7 mutant mice due to the absence of neutrophil attractant CXCL1 (Li et al., 2002). MMPs can act as both pro-inflammatory by recruiting inflammatory cells (Haro et al., 2000) and anti- inflammatory by speeding up clearing and removal of inflammatory cells (Kumagai et al., 1999). MMP-12 contributes to emphysema (Morris et al., 2003) while MMP-9 is pro-inflammatory in skin (Liu et al., 2005). MMP-8 protects againt skin inflammation (Balbin et al., 2003) and MMP-2 provided similar anti-inflammatory protection in brain and spinal cord (Esparza et al., 2004). Thus, MMPs regulate inflammation by controlling or inducing it depending on cellular microenvironment.

MMPs in pathology

Due to the inherent and primary proteolytic function of MMPs, much research has been focused on the role of MMPs in diseases that involve the breakdown of connective tissues like rheumatoid arthritis (RA), osteoarthritis, periodontitis, inflammation and even cancer. During inflammation, leukocytes, particularly, macrophages synthesize and store these MMPs. The MMPs released from macrophages help in the migration, invasion and extravasation of these leukocytes into the tissues thereby causing tissue damage. In addition, there is a production of immunogenic fragments of otherwise normal proteins that catalyzes auto-immune response (Opdenakker and Van Damme, 1992). MMPs are involved in gastric ulcerations and impaired intestinal wounds (Vaalamo et al., 1998). Normal human brain has very low levels of MMPs but these enzymes are significantly elevated in various neurological disorders of CNS (Yong et al., 2001). The high levels of MMPs can induce neuro-inflammation, demyelination of neurons (multiple sclerosis, MS) and disruption of blood-brain barrier with serious consequences (reviewed by Yong et al, 2001; Kieseier et al 1999). Several MMPs are found to be expressed at elevated levels in patients suffering from auto-immune encephalitis (EAE), particularly MMP-2 and MMP-9 double null mice resulted in inability to induce EAE (Agarwal et al., 2006). Due to excessive degradation of ECM, MMPs also contribute to pathologies arising from weakening of matrix such as abdominal aortic aneurysm (AAA) (Aziz and

Kuivaniemi, 2007) and dialated cardiomyopathy, epidermolysis bullosa (Thompson and Parks, 1996).

The requirement of proteolytic enzymes in invasive tumor growth was recognized even before MMPs were discovered (Gersh and Catchpole, 1949). The findings from studies conducted by Gersh and Catchpole showed that fibroblasts secretions are important for tumor growth and invasion. Subsequently, these secretions were characterized and identified as hyaluronidases, serine proteases and MMPs. The critical role of MMPs, specifically type-IV collagenase, in tumor invasion and metastasis was first shown by Lance Liotta (Liotta et al., 1980). The tissue breakdown and remodeling function of MMPs helps in events such as tumor invasion, intravasation into circulation, extravasation, and migration to metastatic sites and angiogenesis (Folgueras et al., 2004; Deryugina et al., 2006). MMPs are over-expressed in a wide range of malignancies with strong correlation between their over-expression and tumor aggressiveness and prognosis (Egeblad and Werb, 2002). Studies underlining the significance of MMPs in cancer are based on knock-out mouse models. MMP-7 null mice show reduced tumor formation (Wilson et al., 1997) while MMP-9 deficient mice showed decreased melanoma metastasis (Masson et al., 1998). MMP-2 deficient mice show reduced melanoma tumor progression and angiogenesis (Itoh et al., 1998). Elevated levels of MMP-2 and MMP-9 contribute to accelerated tumor growth and progression in oral (Ikebe et al.,

1999), lung (Kodate et al., 1997), bladder (Papathoma et al., 2000) and ovarian cancer (Schmalfeldt et al., 2001). Increased levels of MT-MMP correlate with tumor aggressiveness in laryngeal (Du et al., 1999) and esophageal cancer (Etoh et al., 2000). Several studies show that insertion of a guanine (G) nucleotide at position -1607 in the MMP-1 promoter creates a new ETS binding site thereby increasing the transcription of the gene (Rutter et al., 1998). Presence of this 2G nucleotide has been shown to increase the promoter activity of MMP-1 in GBM (McCready et al., 2005) and invasion in colorectal cancer (Ghilardi et al., 2001) and melanoma (Ye et al., 2001). Increased expreesion of MMP-2 and MMP-9 are associated with low survival and increased aggressiveness in brain tumors (Jaalijnoja et al., 2000; Choe et al., 2002).

The above findings emphasized a dire need to develop and test clinically useful MMP antagonists. A large number of MMP inhibitors (MMPIs) are being tested in various cancers in all the three phases of clinical trials (Sideras et al., 2006; Robbins et al., 2008). The first of the MMPIs, Batimastat (BB-94) and Marimastat (BB-2516) were pseudopeptide derivatives that mimic the structure of collagen and blocked MMP activity by competing for the susbtrate-binding site. However, BB-94 had low water-solubility and was not available orally. BB-2516 affected muculoskeletal systems due to off-target effects (Saghatelian et al., 2004). Similar musculoskeletal toxicity has been observed in new MMPIs such as prinomastat and BMS-275291 (Hidalgo et al., 2001; Miller et al., 2004). The

clinical trial results have been found to be disappointing either due to side-effects even at low dose of drugs or the drug was administered at an advanced stage of cancer where MMP inhibition would no longer improve the survival (Robbins et al., 2008).

1.7.4 Regulation of MMPs

To perform the functions during normal physiology and pathological conditions, MMPs are tightly regulated at the transcriptional and translational levels. In addition, the function of MMPs is regulated at protein levels by the presence of endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Hormones, oncogenes, growth factors and cytokines influence the transcription of most of MMP genes by interacting with AP-1 sites present in the promoter region of MMP genes (Benbow and Brinckerhoff, 1997). Some MMPs (MMP-1, -2, -3, -9 and -12) have single-nucleotide polymorphisms that generate an extra ETS binding element site. This site acts synergistically to activate the transcription of these genes (Rutter et al., 1998). Post-transcriptional regulation includes stabilization of mRNA of MMP genes by phorbol esters, EGF and PDGF (Vincenti et al., 2001). Most of the members of MMP family, collagenases, gelatinases and stromelysins are secreted as zymogens. In the ECM, these zymogens are cleaved by other proteases, such as plasmin or by other already activated MMPs (Woessner and Nagase 2000). The TIMPs reversibly inhibit

MMPs by binding in 1:1 stoichiometry (reviewed by Sternlicht and Werb 2001). Other endogenous inhibitors of MMPs are α-macroglobulins that bind to MMPs forming α-macroglobulins/MMP complexes that are cleared by scavenger receptor-mediated endocytosis thereby inhibiting MMPs irreversibly (Sottrup-Jensen and Birkedal-Hansen, 1989).

1.8 Matrix Metalloproteinase-1

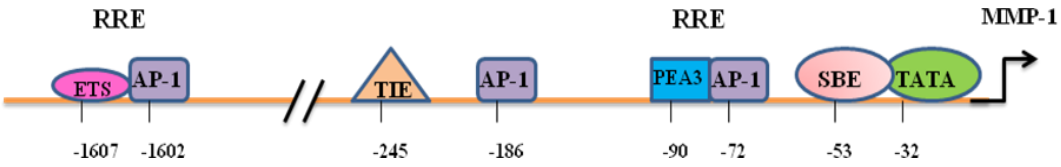
Human fibroblast collagenase (MMP-1) was the first MMP to be discovered (Gross and Lapiere, 1962), purified and cloned (Bauer, Eisen and Jeffrey, 1970; Goldberg et al., 1986). MMP-1 is also known as interstitial collagenase, collagenase-1 or fibroblast collagenase with an official EC number 3.4.24.7. The human MMP-1 gene is located on chromosome 11q22.2-22.3 and has 10 exons. It is tightly linked to a cluster of eight other MMPs including MMP-3, -7, -8, -10, -12, -13 -20 and -27 (Puente et al., 2003). Human MMP-1 is secreted as a proenzyme with 52 kDa as major form and 57kDa as minor glycosylated form. The cleavage of this pro-peptide yields an active and functional MMP-1 with 42 kDa and 47 kDa in sizes respectively (Wilhelm et al., 1986). Structurally, MMP-1 has minimal domain structure that has a hydrophobic pre-domain directing it to endoplasmic reticulum (ER) for secretion. The pro-peptide has a cysteine residue at 73 position in a conserved region PRCGVPD bound covalently through a-SH group to the zinc atom in the catalytic site. The

enzyme becomes activated when this cyteine residue is displaced (cysteine-switch) by proteolytic cleavage or disruption by chemicals (Sprigman et al., 1990). The catalytic domain contains a catalytic zinc bound in the sequence HELGHXXGXXH by three His residues (Li et al., 1995). The crystal structure of catalytic domain has five strands of β-sheet and three α-helices (Li et al., 1995). The catalytic domain is connected by a linker region to a carboxy terminus hemopexin domain. The hemopexin domain helps in enzyme substrate- binding specificity for collagen and interaction with other proteins (Li et al., 1995).

The expression of MMP-1 is low during normal physiology but is elevated when active tissue remodeling is needed. Various growth factors stimulate the expression of MMP-1 by up-regulating signal transduction pathways that converge to activate AP-1 (activator protein-1) transcription factors. Mitogen activated protein kinase (MAPK) pathways activate ERK1/2, JNK and p38 protein to induce the expression of AP-1 proteins. These AP-1 proteins regulate the expression of various genes involved in proliferation, development, differentiation and tumor progression (Karin et al., 1997). The MMP-1 promoter conatins a TATA box at approximately -30bp, and an AP-1 site at -72 with respect to the transcription initiation site (**Figure 1.6**).

Figure 1.6 Regulatory elements of MMP-1 promoter (Simplistic adaptation from Ala-Raho and Kahari, 2005)

The figure shows important elements in the MMP-1 promoter that are discussed in this work. Activator protein-1 (AP-1), nuclear factor κB (NF-κb), polyoma virus enhancer activator-3 (PEA3), STAT binding element (SBE), TATA box (TATA), TGF-β inhibitory element (TIE) are transcription factor binding sites. An ETS site adjacent to AP-1 trancription factor binding site creates a ras responsive element (RRE) at proximal promoter. In the presence of a single nucleotide polymorphism (SNP) at -1607, another RRE is generated at the distal promoter.


RRE
RRE
MMP-1
ETS
AP-1
TIE
AP-1
PEA3
AP-1
SBE
TATA
-1607
-1602
-245
-186
-90
-72
-53
-32

Another AP-1 site is present in the distal promoter at -186 position. The Fos/Jun dimer binds to the AP-1 site which is adjacent to another cis-acting sequence called PEA-3 (polyoma virus enhancer A binding protein 3) where members of ETS transcription factor family bind (Karin et al., 1997; Sharrocks et al., 1997). AP-1 and ETS transcription factors act synergistically to increase the transcription of MMP-1 (Westermarck et al., 1997). As mentioned earlier, the MMP-1 promoter exhibits single-nucleotide polymorphism (SNP). At position -1607, there is an extra guanine nucleotide generating ETS transcription factor binding site. Together with an adjacent AP-1 site at -1602 position, it forms a ras responsive element (RRE) (Rutter et al., 1998). Oncostatin M enhances expression of MMP-1 by activating STAT transcription factors that bind to SBE and co-operate with AP-1 elements on MMP-1 promoter (Korzus et al., 1997) **(Figure 5).** The p53 protein is a transcription factor that binds to a specific consensus sequence in the promoter of several genes. In addition, it can also interact with proteins to alter their functions. Although, MMP-1 promoter does not have a p53 binding element, it has been shown that wild-type p53 down-regulates MMP-1 transcription (Sun et al., 1999). Subsequently, it was shown that down-regulation of MMP-1 by wild type p53 is due to its interaction with p300 protein present in the transcriptional complex (Sun et al., 2004). The cytokine transforming growth factor-β (TGF- β) suppresses MMP-1 transcription by binding to TGF- β inhibitory element (TIE) present on MMP-1 promoter at

position -245. Mutations in this element result in induction of MMP-1 transcription by tissue polypeptide antigen (TPA) (White et al., 2000).

The human MMP-1 mRNA has an AU-rich element which is important for its stabilization (Vincenti et al., 1994). Activation of p38α results in MMP-1 mRNA stabilization (Reunanen et al., 2002) by activating a zinc-finger protein, tristetraprolin that regulates the stability of AU-rich regions of mRNA (Carballo et al., 2000).

Activation of latent precursor of MMP-1 by pro-domain proteolytic cleavage takes place in a stepwise manner requiring interaction with plasminogen activator urokinase and stromelysin (Suzuki et al., 1990). The serine proteases cleave in the middle of pro-domain of MMP-1 generating a 46-kDa intermediate form that is further cleaved between Val-67 and Met-68 residues forming a 43kDa form. It needs to be cleaved further for a full activation and this requires cleavage by MMP-3, -10 or MMP-7 between Gln-80 and Phe-81 (Ito et al., 1998; Suzuki et al., 1990; Imai et al., 1995).

MMP-1 has been found to be over-expressed in normal physiological processes like embryonic development and wound healing (McGowan et al., 1994; Ravanti et al., 2000). In addition, it is also found be elevated in pathological states such as ulcers (Vaalamo et al., 1998) and a wide range of

cancers of breast (Wang et al., 1997), colon, stomach, ovary (Murray et al., 1996, 1998), pancreas (Ito et al., 1999) and skin (Nikkola et al., 2002). The presence of SNP favors the growth and progression of ovarian and colorectal cancers (Kanamori et al., 1999; Ghilardi et al., 2001). MMP-1 has also been found to be over-expressed in brain neoplasms (Nakagawa et al., 1994; Nakano et al., 1995; McCready et al., 2005).

1.9 Epidermal Growth Factor Receptor Signaling

For a cell to function normally, it requires nutrients and growth factors for maintainance and proliferation. Growth factors communicate with the cell by binding to transmembrane receptors presented on the surface of the cell. This type of signaling is crucial to the development and survival of a normal cell during typical physiological conditions. In a cancer setting, such growth signaling mechanisms go awry; the tight control on the activation and suppression of signaling mediated by these growth factors becomes defective. The cells become self-sufficient and constitutively produce these growth factors or they evade mechanisms that inhibit the growth signaling (Hanahan and Weinberg, 2000).

Epidermal Growth Factor Receptor (EGFR) is an oncogene that is involved in the regulation of several cellular processes mainly cell proliferation, survival and migration. Altered regulation of EGFR activity is an important event

that leads to formation and progression of various human malignancies in brain, lung, breast, ovary, pancreas and prostate (Hynes and Stern 1994; Saloman DS et al., 1995).

Structurally, EGFR is a 170kDa membrane spanning protein consisting of 1186 amino acids. The receptor has an extracellular ligand binding domain, a transmembrane domain and a cytoplasmic tyrosine kinase domain (Ogiso H et al., 2002; Schlessinger J 2002).

The ErbB or Epidermal Growth Factor (EGF) family of receptor tyrosine kinases (RTKs) include four members- EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. During normal physiological conditions, the activation of EGFR is controlled by the expression and availability of its ligands, EGF –related peptide growth factor family (reviewed by Riese and Stern 1998). The ligands for these receptors have been divided into three groups- first group includes EGF, Amphiregulin (AR) and transforming growth factor-α (TGF-α) that bind specifically to the EGFR/ErbB1. The second group consists of heparin-binding EGF (HB-EGF) and epiregulin (EPR) that bind to both ErbB1 and ErbB4. The third group has neuroregulins (NRG) that bind to ErbB4 (NRG-3 and -4) and to both ErbB3 and ErbB4 (NRG-1 and -2). There is no direct ligand discovered, so far, for ErbB2 suggesting that it is a co-receptor (Holbro T et al., 2003). Among its ligands, EGF is an important regulatory factor that is present in

abundance in various human cancers, either due to autocrine or paracrine signaling pathways.

Binding of EGF to its receptor, EGFR, leads to receptor homo- or hetero-dimerization (Olayioye MA et al., 2000) followed by activation of the receptor's tyrosine kinase domain through trans-tyrosine phosphorylation. The activated receptor kinase phosphorylates the tyrosine residues on the C-terminal tail of the ErbB receptors. These phosphorylated sites allow the binding of proteins with src-homology (SH2) domains such as Grb2, shc and Nck. These are adaptor proteins that have intracellular docking sites for other proteins such as serine threonine kinases that further activate other serine threonine kinases and transcription factors (Olayioye MA et al., 2000). Thus, signaling from EGFR leads to an amplification of several signaling pathways that alter protein function and gene transcription favourable for oncogenesis. The best studied EGFR downstream signaling pathways are RAS-RAF-MEK-ERK and PI3K-AKT pathways. During normal conditions, the EGFR signaling is transient and is terminated either by phophotyrosine phosphatases or after the receptor is endocytozed and binds to c-cbl protein that targets it to degradatory pathways (Lipkowitz et al., 2003; Muthuswamy et al., 1999).

EGFR mediated signaling pathways

1.9.1 MAPK pathway.

Epidermal growth factor receptor stimulation leads to activation of several signaling pathways including the mitogen activated protein kinase (MAPK) pathway. The MAPKs are a large family of serine-threonine kinases that include extracellular signal regulated kinases (ERKs), Stress activated protein kinases (SAPK) or c-jun terminal kinases (JNKs) and p38 mitogen-activated protein kinases. These kinases are phosphorylated and activated by MAPK kinases (MKKs) which in turn are activated by MKK kinases (MKKKs) (Johnson GL et al 2005). Upon EGF stimulation, the EGFR binds to adaptor proteins Grb2 or shc and recruits son-of-sevenless (SOS) protein (Wu J et al 1993). The SOS protein at the plasma membrane causes a small G protein RAS to release its GDP and exchange it for GTP thereby activating it. The activated RAS targets its downstream protein, Raf-1, which is a MKKK. Raf-1 kinase activates and phosphorylates MKK, MEK1/2, that in turn activates ERK1/2. ERK1/2 is a MAPK and it activates various other proteins such as ribosomal S6 protein kinase (Rsk), MSK, $cPLA_2$, c-Myc, Ets-2 and Elk leading to the increases gene transcription of anti-apoptotic proteins (Bcl-2 and inhibitor of apoptosis, IAPs) (reviewed in Henson and Gibson, 2006).

1.9.2 PI3 kinase/AKT pathway.

Phosphatidylinositol- 3 kinase (PI3-kinase) protein has an SH2 domain that is needed for interaction with the EGFR after its stimulation. PI3-kinase is recruited to the plasma membrane where it catalyzes the transfer of a phosphate group from ATP to phophatidylinositol generating a 3'- phophatidylinositol phosphate (PIP3). This process is reversed by phosphatase and tensin homolog protein (PTEN). PIP3 binds to proteins with pleckstrin homology (PH) domains. AKT (also called as protein kinase B, PKB) is a serine-threonine kinase that has PH domain and binds to PIP3 and gets phosphorylated. AKT has been regarded a key player in the survival pathway of a cell by acting on its downstream targets that are categorized into apoptotic proteins, transcription factors and protein kinases. AKT activates transcription factors- NFκB, CREB and HIF-1α to increase the transcription of anti-apoptotic proteins. AKT phosphorylates inhibitor of NFκB kinase (IKKα/β), which phosphorylates and inactivates IκB. IκB dissociates itself from its NFκB bound state and gets degraded releasing NFκB for nuclear translocation (Song et al., 2005). EGFR inhibition sensitizes cells to apoptosis by de-activating AKT (Longva et al., 2005). Glycogen synthase kinase- 3 is AKT downstream target that gets inactivated by phosphorylation leading to reduced transcriptional activity and metabolism (reviewed by Woodgett, 2005). AKT phosphorylates and activates mTOR, mammalian target of rapamycin, a serine-threonine kinase. mTOR activates ribosomal S6 kinase and eukaryotic initiation factor 4E- binding

protein 1 (4E-BP1) leading to an overall increases survival and increased translational activity in the cell (Sun et al., 2005).

Evidence of cross talk signaling between PI3K and MAPK has been reported. Hyper-phosphorylated AKT also phosphorylates and inactivates Raf thereby attenuating MAPK singaling (Zimmerman and Moelling, 1999). In addition, PI3K also activates RAS leading to the activation of MAPK pathway (Jun et al., 1999).

1.10 EGFR biology in GBM

Published findings show that EGF stimulates tumor growth, invasion and migration in an *in-vitro* glioma model (Lund-Johansen et al., 1990). EGFR over-expression is associated with resistance to radiation in malignant GBM cells (Chakravarti et al., 2002) and poor response to radiation therapy in patients with GBM (Barker et al., 2001). Radiation leads to EGFR activation and upregulation of MAPK and PI3K pathways which then lead to an increased cytoprotective response (Schmidt-Ullrich et al., 1997; Dent et al., 2003) and accelerated cell proliferation (Carter et al., 1998). Inhibition of EGFR radio-sensitizes and induces apoptosis in malignant glioma (Birgit et al., 2008) leads to decreased invasion, inhibition of U251 glioma cell growth (Stea et al., 2003) and cytotoxicity in U87 glioma cell line (Yamoutpour et al., 2008). The interruption

of MAPK and PI3K/Akt signaling pathways may provide mechanisms for inhibition of tumor growth and overall survival.

AIMS

The focus of this dissertation was to examine the function of MMP-1 in GBMs. Although, the primary substrate for MMP-1, collagen, is lacking in GBMs, there is a significantly increased expression of MMP-1 in GBM as compared to normal physiology. In recent years, mounting evidence shows that MMPs do not just degrade ECM; they perform important functions by cleaving bio-active molecules present in the ECM to activate various oncogenic signaling pathways. Extensive literature suggests the involvement of MMP-1 in various pathologies including cancer. Therefore, the regulation of this particular enzyme is paramount for better therapy. In GBMs, a majority of patients have EGFR signaling pathway abnormalities. We wanted to examine the correlation between EGFR-mediated tumor aggressiveness and MMP-1 expression. Chapter 3 focuses on the regulation of MMP-1 by EGFR and the signaling pathways associated with it.

CHAPTER 2

Matrix Metalloproteinase-1 over-expression in human glioblastomas: Implications in glioma cell invasion

Anand M, Mullet E, McCready J, Abd-Elfattah N, Fuller CE, Broaddus WC, and Fillmore HL

2.1 Abstract

Glioblastoma Multiforme (GBM) is the most common form of primary malignant brain tumor with a median survival of approximately one year post-diagnosis. The highly invasive characteristic of GBM, an important factor contributing to the failure of therapies, involves the action of proteolytic enzymes such as matrix metalloproteinases (MMPs). MMP-1 is a key enzyme that leads to invasion in many cancers by degrading extra-cellular matrix (ECM) proteins and cleaving latent bioactive molecules that aid in the up-regulation of pro-invasive proteins. We examined the expression of MMP-1 in human GBM tissue specimens and its function in GBM cell lines. The protein levels of MMP-1 in GBM patient samples were analyzed using Enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. Down-regulation of MMP-1 was performed in T98G glioma cells by transient transfection with MMP-1 siRNA and MMP-1 over-expression was achieved in U251MG and U87MG cell lines by stable transfection of a MMP-1-pIRES cDNA vector. Invasion and cell proliferation assays were conducted. We observed that MMP-1 protein levels are elevated in human GBM tissue as compared to normal brain. Immunohistochemistry of patient samples indicated that MMP-1 protein is present in both glioma cells and endothelial cells. Inhibition of MMP-1 expression in T98G cells by siRNA resulted in a significant decrease in invasion compared to controls. Glioma cells engineered to stably over-express MMP-1 showed a significant increase in

invasion compared to controls. Thus, we can conclude that MMP-1 is over-expressed in human glioma tissue and can influence glioma cell invasion *in vitro* suggesting its importance in glioma biology.

2.2 Introduction

Despite significant improvements in the diagnosis and treatment of patients with glioblastoma multiforme (GBM), this primary brain tumor remains essentially incurable. Initial treatment usually includes surgical resection of the tumor mass, followed by radiation and chemotherapy. In virtually all cases the tumor recurs, usually near the margins of the previous resection, often in a form that is more resistant to subsequent therapies. A key feature that underlies the malignant behavior of this disease is the ability of glioma cells to aggressively infiltrate surrounding brain tissue (Zhu and Parada, 2002; Giese et al., 2003; Rao et al., 2003). The presence of invasive cells in the brain surrounding the primary tumor explains the tendency of these tumors to recur at these margins. Growth factors, extracellular matrix molecules, and proteases are secreted from both tumor and normal brain cells, and are likely to interact in complex ways to contribute to the highly invasive behavior of GBM cells. Tumor cell invasion involves interactions with and degradation of molecules of the extracellular matrix followed by active cell migration. Matrix metalloproteinases (MMPs) are important proteolytic enzymes that have been implicated in the invasion process

in a wide variety of cancers (Egeblad and Werb, 2002). With over 25 members, MMPs are classified as stromelysins (that cleave fibronectin and proteoglycans), interstitial collagenases (that cleave triple helical regions of fibrillar collagens: Types I, II, III, VII, VIII and X) and gelatinases (that cleave denatured collagen, fibronectin, elastin and collagens: Types IV, V, VI, VII and X). MMPs are tightly regulated at the transcriptional and translational levels and once secreted from tumor or host cells, they can activate other proteases.

Accumulating evidence suggests a correlation between elevated expression of MMPs and glioma progression. Published findings show that MMP-2 (Gondi et al., 2009; Kargiotis et al., 2008; Yamamoto et al., 1996; Lampert et al., 1998; Vince et al., 1999), MMP-7 (Vince et al., 1999), MMP-9 (Ezhilarasan et al., 2009; Lakka et al., 2005; 2002), MMP-14, MMP-15 (Fillmore et al., 2001) and MMP-16 (Xia et al., 2009) are involved in brain tumor biology. Of the matrix metalloproteinase family, MMP-2, MMP-14 and MMP-9 have been the most extensively studied in brain tumors whereas MMP-1 has been less characterized. Limiting the role of MMP-1 to collagen breakdown may have led to a lack of study in the brain, where there are not significant quantities of collagen type I. MMP-1, also known as collagenase I, was first described by Jerome Gross in the early 1960s (Gross and Lapiere 1962; McCawley et al., 2001). In addition to cleaving type I collagen, MMP-1 also activates MMP-2 (Vihinen et al., 2002) and stimulates MMP-9 release from cultured neural cells

and macrophages (Conant et al., 2002). Other than type I collagen, MMP-1 can cleave aggrecan, laminin, tenascin and fibrin. It is also known to cleave latent forms of cytokines such as pro-TNF-α (McCawley et al., 2001). We have reported that there is a significant increase in MMP-1 mRNA levels in tumor samples from patients with GBM relative to normal brain (McCready et al., 2005). MMP-1, which is not typically expressed in normal brain (McCready et al., 2005), has been shown to be elevated in glioma (Hodgson et al., 2009) and correlates with tumor grade and survival time (Stojic et al., 2008; Nakano et al., 1995). Herein we use an ELISA assay and immunohistochemical techniques to examine the expression of MMP-1 protein in human glioma tissue. To investigate the role of MMP-1 in glioma cell invasion, we have performed Matrigel-invasion assays in glioma cell lines that were manipulated by stable over-expression and transient siRNA-mediated inhibition of MMP-1. Results demonstrate that MMP-1 expression promotes and MMP-1 suppression inhibits glioma cell invasion.

2.3 Materials and Methods

Cell Culture

Human GBM cell lines (T98G, U251MG and U87MG) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM Invitrogen, Carlsbad,

CA, USA) supplemented with 4.5 g/l D-glucose, 1% penicillin/streptomycin (Invitrogen) and 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, West Sacramento, CA, USA). Cells were incubated in a humidified incubator at 37°C with 5% CO_2 in Nunclon 75cm^2 culture flasks (Nunc, Germany) and harvested with 0.25% Tryspin-EDTA (Invitrogen).

Tissue samples

Tumor tissue was obtained at the time of craniotomy and was dissected free of adventitial material, snap-frozen in liquid nitrogen, and maintained in a tissue bank at -80^{0}C, in accordance with a protocol approved by the VCU Institutional Review Board. Confirmation of diagnosis of GBM was determined by a staff neuropathologist.

Enzyme-linked immunosorbent assay

Total protein was extracted from 6 normal brain samples and 26 glioma samples using Tissue Protein Extraction Reagent (T-PER, Pierce, IL) and quantified using the Coomassie Plus Protein Assay Reagent Kit (Pierce, IL). 10μg of total protein was subjected to a sandwich enzyme immunoassay. Capture and detection antibodies were used (CLMABMMP1, ACL2MMP1, Cedar Lane, Canada) and incubation was carried out at room temperature for 2 hours. After the incubation, the plate was washed 5 times with PBS followed by addition of tetramethylbenzidine. The plate was incubated in the dark at room temperature

for 30 minutes, and the reaction was stopped by addition of 2.5N H_2SO_4. The absorbance at 450 nm was measured by a microplate reader (Molecular Devices, Inc., Sunnyvale CA).

Immunohistochemistry

The MMP-1 immunohistochemical (IHC) staining was performed using a rabbit anti-MMP-1 antibody (Abcam catalog# ab38929, Cambridge MA) at a dilution of 1:100. Slides were deparaffinized through 3 changes of Xylene for 5 minutes each and rehydrated through 2 changes of 100% reagent alcohol for 3 minutes each, 1 change of 95% reagent alcohol for 3 minutes, and 1 change of 80% reagent alcohol for 3 minutes. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide at room temperature for 5 minutes. The IHC stain was performed using Heat Induced Epitope Retrieval (HIER), consisting of a 20 minute incubation in Target Retrieval solution, pH 6 (Dako Corporation, Carpinteria CA) and a 20 minute cooling period. Slides were placed on an Autostainer Plus^{TM} (Dako Corporation, Carpinteria CA), on which all subsequent incubations were performed at room temperature and all washes consisted of a rinse with Wash Buffer (Dako Corporation, Carpinteria CA). Nonspecific binding sites were blocked with Serum Free Universal Protein Block (Dako Corporation, Carpinteria CA) for 5 minutes. Slides were then washed and incubated with the primary antibody at the above-mentioned dilution in Antibody Diluent with Background Reducing Agents (Dako Corporation, Carpinteria CA)

for 1 hour. An incubation with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), dilution 1:500 in previously noted antibody diluent was followed by a wash, and subsequent incubation in Envision+^{TM} Dual Link (Dako Corporation, Carpinteria CA) for 30 minutes. Slides were washed again and incubated for 10 minutes in DAB+ (diaminobenzidine--Dako Corporation, Carpinteria CA). Slides were removed from the Autostainer Plus^{TM} and counterstained with Gills III Hematoxylin (Poly Scientific, Bay Shore, NY), blued in tap water, rinsed in deionized water, dehydrated through 1 change of 80% reagent alcohol for 3 minutes, 1 change of 95% reagent alcohol for 3 minutes, and 3 changes of 100% reagent alcohol for 3 minutes each. The dehydration was followed with 3 clearing changes of Xylene for 5 minutes each and coverslipped using Tissue Tek Glas Mounting Media (Sakura, Torrance, CA) and 24x55 #1 coverglasses (Cardinal Health, McGaw Park, IL). Images were obtained using an Olympus DP72 digital camera (Olympus America Inc, Center Valley, PA) mounted on a Nikon Eclipse E600 microscope (Nikon Instruments Inc, Melville, NY).

MMP-1 siRNA transient transfection

T98G cells were transfected with *Silencer* pre-designed siRNAs against MMP-1: siRNA ID 1157 sense, GGUAUGAUGAAUAUAAACGtt (Ambion, Austin, TX, USA), targeting between 1265 to 1285 bases in the human MMP-1 transcript. The cells were plated in complete media (without antibiotics) in six-

well plates at a density of 200,000 cells per well. At 80% confluence, media were removed from the cells and the wells were washed with PBS followed by addition of 800µl per well of Opti-MEM I reduced serum medium (Invitrogen). Oligofectamine transfection reagent, Opti-MEM I and siRNA or scrambled RNA (at concentrations of 30nM) mixtures were made and incubated at room temperature for 20 min. Then cells were treated with reaction mixtures of scrambled siRNA and predesigned siRNA against MMP-1. The six well plates were kept on a shaker at 37°C for 6 hours. Opti-MEM I supplemented with 30% FBS was added (500 µl per well) for overnight incubation. Complete media were added the next day (500µl per well). At the end of 48 hours, media were replaced with serum-free media for overnight incubation at 37°C. Proteins were extracted from conditioned media and cells after the overnight incubation.

Stable over-expression of MMP-1

U251MG 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. One million cells were transfected with 1µg DNA using the Oligofectamine transfection method according to the manufacturer's protocol (Invitrogen). Three days after transfection, medium containing 500µg per ml Geneticin (G418, GIBCO) was added to the U251-vector control and MMP-1 over-expressing transfectants. The Geneticin–resistant cells were selected and grown for further experiments. Afterwards, these cells were stably maintained in

complete DMEM supplemented with 400μg per ml Geneticin. Over-expression of MMP-1 was confirmed by immunoblot studies as shown in experimental results below.

Immunoblot

Cell lysates and conditioned media were collected from the GBM cell lines-T98G, U251MG and U87MG. In these experiments, the conditioned media were collected and concentrated from a volume of 4ml to 250μl using Amicon centrifugal filters (Millipore). For preparation of cell lysates, the cells were treated with ice cold RIPA buffer (150 mM NaCl, 50mM Tris, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Calbiochem). The lysates were centrifuged at 14000 rpm for 20 min after shearing with 26 gauge needles. Protein concentrations were determined using DC Protein Assay (Bio-Rad, Hercules, CA, USA)

Expression of MMP-1 protein levels was determined by immunoblot analysis. Proteins isolated from conditioned media (10ug) and cell lysates (30μg) were separated in 4-12% polyacrylamide –bis-Tris gels (Invitrogen, Carlsbad, USA) and transferred to a 0.45μm nitrocellulose membrane (Invitrogen, Carlsbad, USA). After blocking in 5% milk in TBST (50mM Tris base, 150mM NaCl, 0.05% (v/v) Tween 20), the membranes were incubated with mouse monoclonal anti-MMP-1 antibody (R & D Systems, Minneapolis, MN, USA at 1:200 dilution)

in the blocking solution. Rabbit polyclonal anti-cyclophilin A antibody (Millipore, Billerica, MA, USA) was used to quantify any variability in loading onto the polyacrylamide gels. After washing the membranes in TBST, they were incubated with horseradish peroxidase –conjugated secondary antibodies: goat anti rabbit IgG and goat anti mouse IgG, (Rockland Immunochemicals, Gilbertsville PA, USA) both diluted 1:5000 in 5% milk-TBST solution. Blots were visualized with exposure of autoradiographic film using an enhanced chemiluminescent system (ECL, GE Healthcare). The developed films were scanned and densitometry was performed using ImageJ.

Matrigel Invasion Assays

Invasiveness of GBM cell lines was determined using transwell inserts (Corning Incorporated, USA) with 8μm pores. The inserts were coated with growth factor reduced Matrigel (BD Biosciences, CA, USA) at a concentration of 100 μg per filter diluted in cold serum-free DMEM for 30 minutes at 37°C incubator. 600 μl of 10% serum containing medium was added to the bottom chamber. The cells at a density of $1x10^6$ cells per ml were added to the top chamber and incubated at 37°C for 48 hours. At the end of incubation, the cells that passed through the Matrigel into the bottom chamber were counted under the microscope from four random fields per transwell. Three replicates for each experimental condition were analyzed.

Cell Proliferation Assays

Cell proliferation was measured using the CellTiter-Glo luminescent Cell Viability ATP assay (Promega). 1000 cells per 100μL media were plated in 6 replicates per condition in white-walled 96 well plates (Corning Life Sciences). At the end of each time point (days 0, 1, 3 and 5), 100 μl per well of Cell Glo solution was added directly to the wells containing cells, with gentle shaking at room temperature for 10 minutes followed by incubation at 4°C for 10 min. A standard curve of known ATP concentrations was generated for each assay. Luminescence was detected using a Fluostar Optima plate reader (BMG Labtech, Durham, NC, USA), and ATP estimated based upon standard curve luminescence. The measurement of number of cells was correlated with the luminescence obtained from serial dilutions of cell-counts from each cell line.

Statistical Analysis

Data were evaluated by comparing the means and SEM of replicate experiments. Data are expressed as the mean and SEM of at least three independent experiments. Statistical analysis was done using an unpaired Student's *t* test. $P < 0.05$ was considered significant.

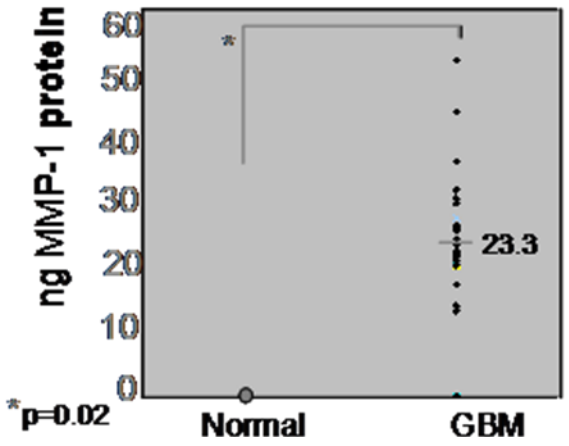
2.4 Results

Matrix metalloproteinase-1 is over-expressed in human GBM tissue specimens

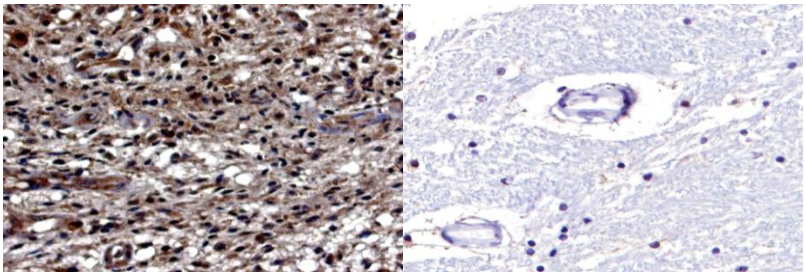
To determine the expression levels of MMP-1 in GBM, we analyzed protein from 6 normal brain samples and 26 GBM patient samples. Of the 26 GBM samples tested, the majority of samples had MMP-1 levels ranging from 12 to 54 ng per 10 μg of total protein and only two had no detectable levels of MMP-1. There were no detectable levels of MMP-1 in normal brain tissue samples (**Figure 2.1A**). To confirm the expression and localization of MMP-1 in GBM patients, we performed immunohistochemistry with paraffin-embedded sections of three human GBM samples using an antibody against MMP-1. Immunostaining confirmed the expression of MMP-1 in all of the GBM tissue samples (**Figure 2.1B**), particularly in the perivascular regions of the tissues. There was no detectable MMP-1 expression in normal brain adjacent to tumor (**Figure 2.1B**).

Figure 2.1 MMP-1 protein expression in GBM. A) MMP-1 protein levels in normal versus GBM patient samples as measured by ELISA. B) MMP-1 expression in three representative GBM paraffin-embedded tissue samples.

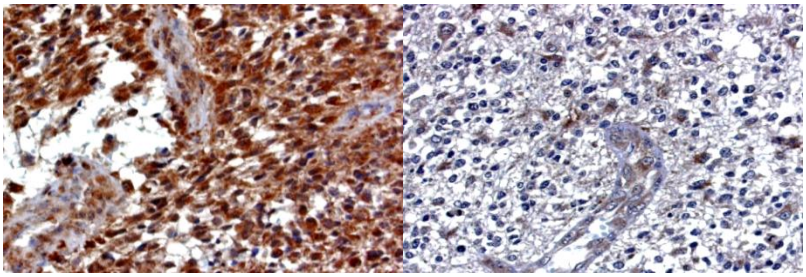
A



B



Normal Brain **GBM #1**

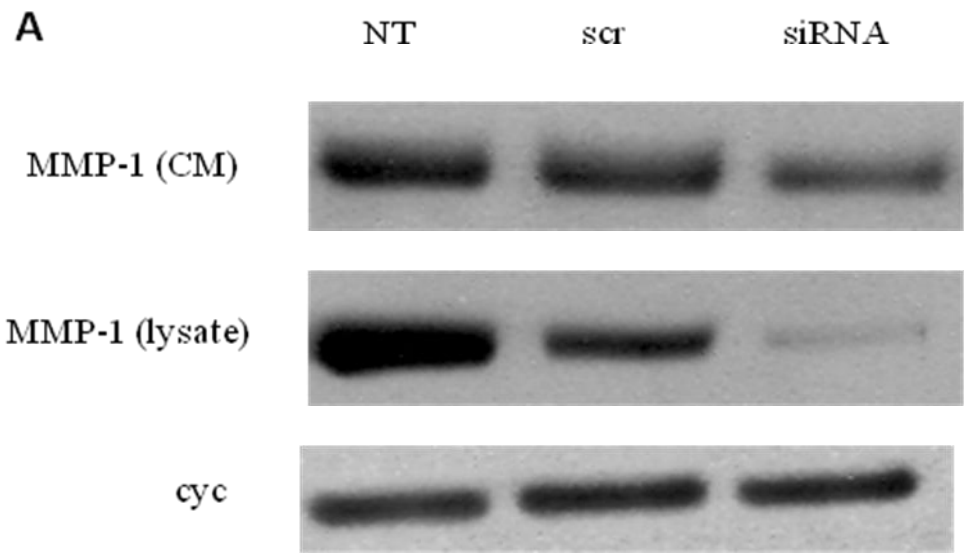


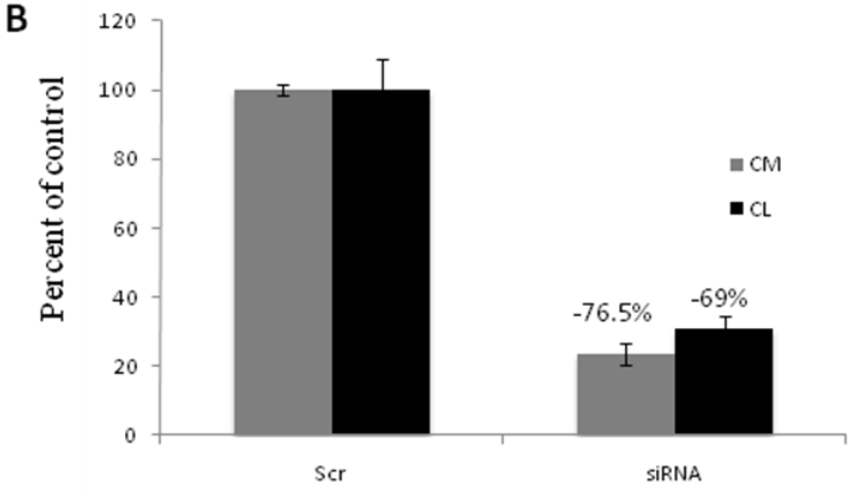
GBM #2 **GBM#3**

MMP-1 inhibition by siRNA leads to a decrease in glioma cell invasion

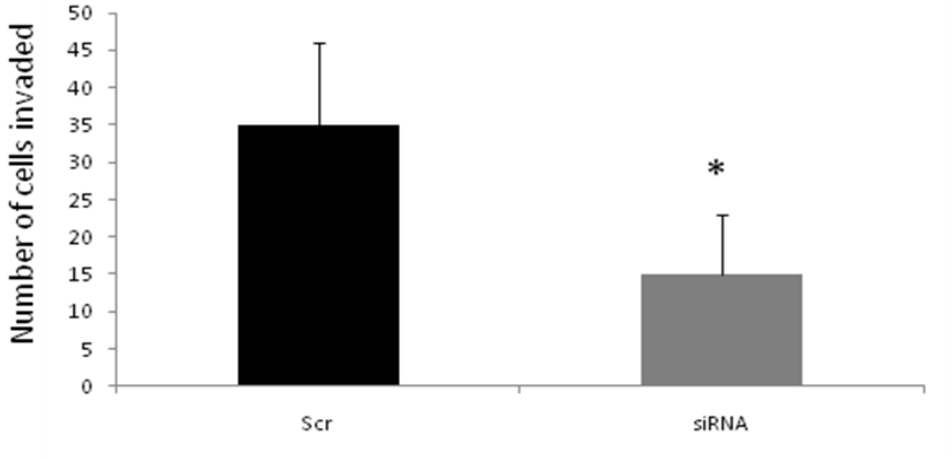
MMP-1 has been established as an important contributor to invasion in other malignancies. To examine if it has a pro-invasive function in glioma, we first used siRNA to specifically inhibit MMP-1. The T98G cell line was used for these inhibition studies due to the presence of a high basal level of MMP-1. Treatment with 30nM MMP-1 siRNA inhibited the expression of MMP-1 in the T98G cell line as observed in both conditioned media and cell lysates (**Figure 2.2A**). Densitometric analysis demonstrates a decrease in MMP-1 immunoreactivity in both conditioned media (-76%) and cell lysates (-69%) (**Figure 2.2B**). In a parallel experiment, invasive capacity of the T98G cells treated with MMP-1 siRNA was examined. There was a significant decrease in glioma invasion in cells that were treated with MMP-1 siRNA compared to scrambled RNA-treated and no treatment controls (**Figure 2.3C**). To determine whether the viability of cells treated with siRNA was affected, we performed growth curves of control and siRNA-treated T98 cells using an ATP-based viability assay (**Figure 2.3D**). There was no difference in ATP detection among the various groups indicating that the siRNA does not affect the *in vitro* growth of T98G cells.

Figure 2.2 Transient inhibition of MMP-1 using siRNA leads to a decrease in glioma invasion. **A)** Representative immunoblot of T98G cell lysates (CL) and conditioned medium (CM) from cells treated with MMP-1 siRNA using Oligofectamine at 30nM concentration in T98G cells, NT (no treatment), Scr (scrambled control), Transfection reagent (Oligofectamine) and MMP-1 siRNA, cyclophilin (cyc) is used as loading control for cell lysates. **B)** Densitometric analysis of conditioned media and cell lysates (normalized to cyclophilin control) from scrambled and siRNA treated samples. Data is representative of three independent experiments and densitometric analysis is conducted across three experiments (n=3). **C)** Matrigel- invasion assay shows the comparison of the number of cells that invaded through a 100 μg matrigel (per filter) in a 48 hour timeperiod between scrambled and MMP-1 siRNA treated T98G cells, * p value < 0.005. **D)** Cell Proliferation Assay performed using Cell-Titer Promega kit in T98G cells treated with scrambled (scr) and MMP-1 siRNA over 0, 1 and 3 days time points show no difference in viability in MMP-1 siRNA treated T98G cells compared to the scrambled treated control T98G cells. Data is mean of three separate experiments (n=3).

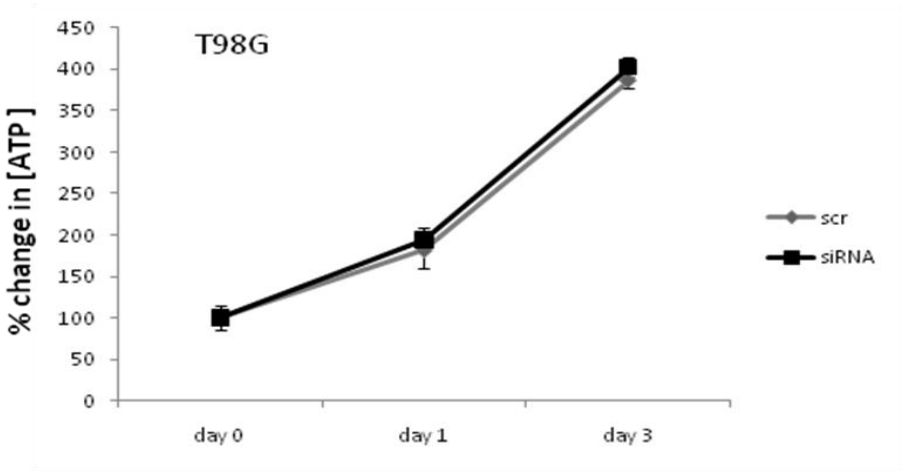

A
NT
scr
siRNA
MMP-1 (CM)
MMP-1 (lysate)
cyc


B
Percent of control
120
100
80
60
40
20
0
CM
CL
-76.5%
-69%
Scr
siRNA

C


Number of cells invaded
50
45
40
35
30
25
20
15
10
5
0
*
Scr
siRNA

D

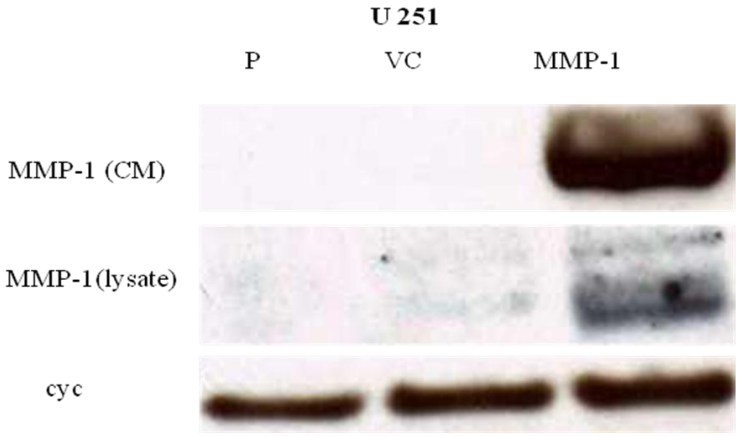

T98G
% change in [ATP]
450
400
350
300
250
200
150
100
50
0
day 0
day 1
day 3
scr
siRNA

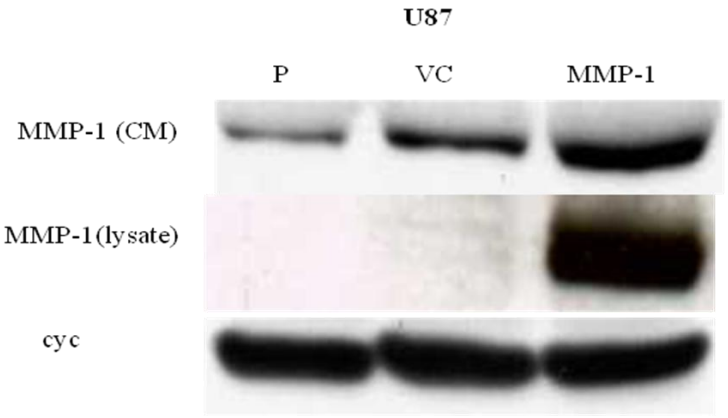
MMP-1 over-expression leads to increased invasion

Based on the results obtained from the inhibition studies we created U251MG and U87MG cell lines that stably over-express MMP-1 (**Figure 2.3A**). In normal culture conditions, these cell lines have low basal expression of MMP-1. Following confirmation of over-expression of MMP-1 by immunoblots, the parental, vector control and MMP-1 over-expressing cells were examined for invasive potential in matrigel-based invasion assays. We found that MMP-1 over-expressing cell lines were significantly more invasive than the controls (**Figure 2.3B**). There was approximately 50% increase in invasion in MMP-1 over-expressing U87 cell lines over U87 vector control ($p < 0.05$). In addition, U251 cells over- expressing MMP-1 were 68% more invasive than the vector controls ($p<0.05$) (**Figure 2.3B**). To determine if the increased invasion was due to an increase in cellular proliferation, we performed *in-vitro* cell proliferation assays with the U251MG and U87MG stably transfected cells along with the controls. We found that there was no observable difference in the proliferation rates among the parental, vector controls and MMP-1 over-expressers (**Figure 2.3C**). These results indicate that MMP-1 increases glioma cell invasion *in vitro* and suggest that MMP-1 may play a role in GBM invasion.

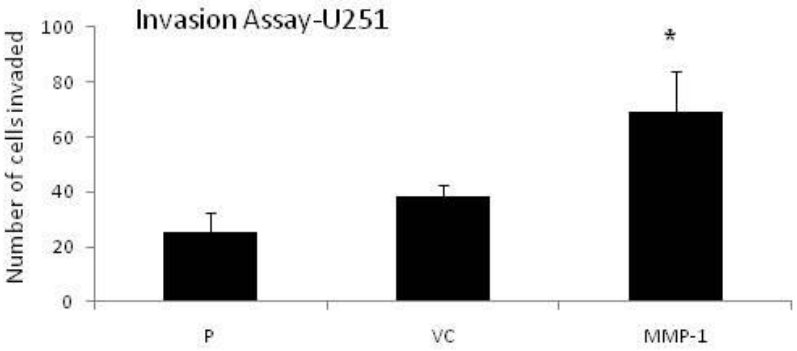
Figure 2.3 A). U251MG and U87MG cells were stably transfected with a pIRES-GFP-MMP-1 cDNA vector with neomycin resistance. Immunoblots confirm the over-expression of MMP-1 in conditioned media and cell lystaes (cyclophilin used as loading control). **B**) Invasion assays using U251MG and U87MG parentals (no treatment), vector control (pIRES-GFP with neomycin resistance) and MMP-1 over-expression were performed. There is a significantly increase in invasion in MMP-1 over-expressing U251MG and U87MG cells as compared to the controls (vector control and parental). Data is representative of three independent experiments, p*<0.05 (U251MG MMP-1 over-expressing cells as compared to the vector-control), p#<0.05 (U87MG MMP-1 over-expressing cells as compared to the vector-control) (n=3). **C)** Cellular proliferation assays were performed using a Cell-Titer Promega kit. No difference was observed in the cell proliferative ability among the three sample groups in each cell line. Data shown is mean of three separate experiments, n=3.

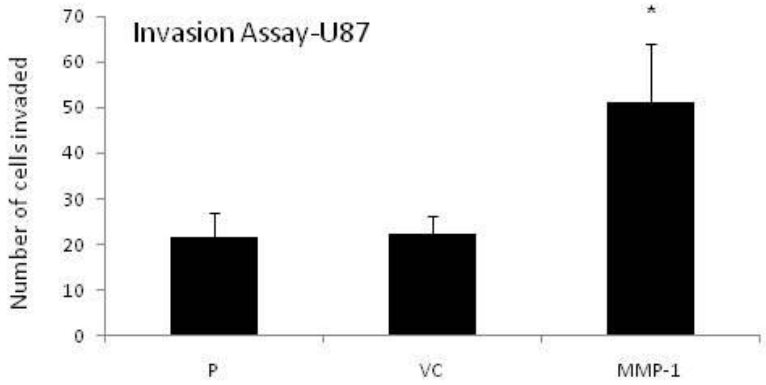
A


U 251
P
VC
MMP-1
MMP-1 (CM)
MMP-1(lysate)
cyc

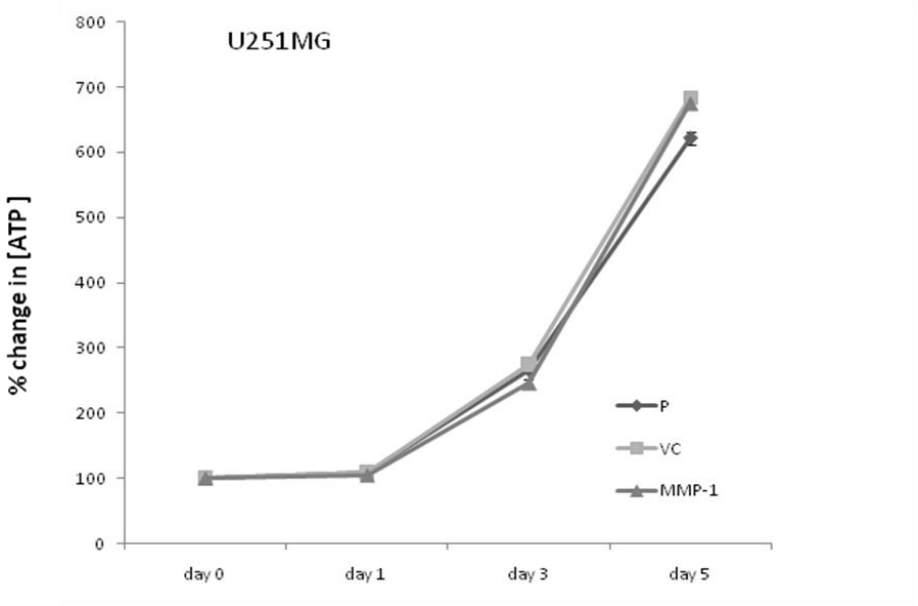

U87
P
VC
MMP-1
MMP-1 (CM)
MMP-1(lysate)
cyc

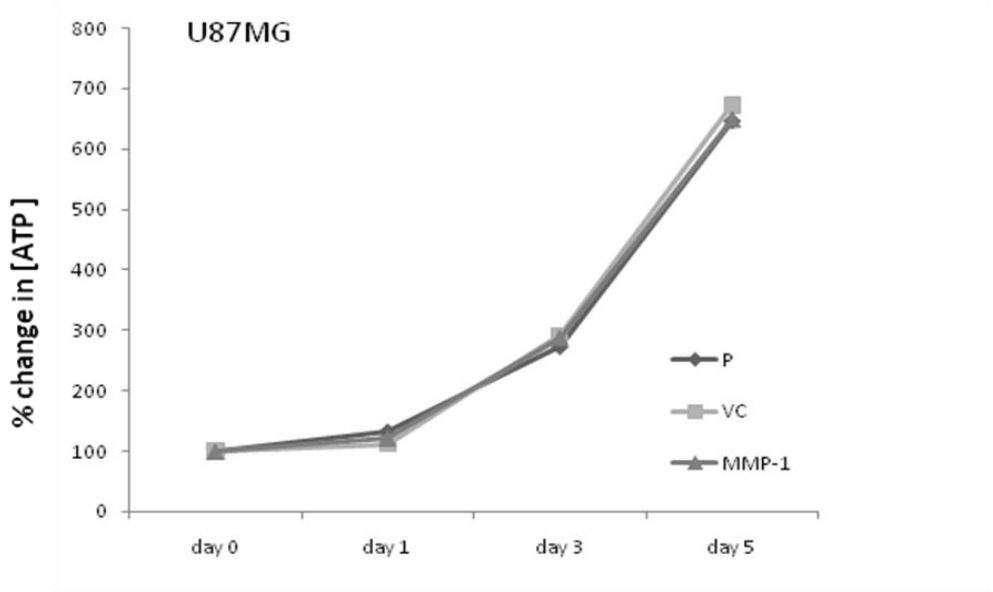
B


Invasion Assay-U251
Number of cells invaded
100
80
60
40
20
0
*
P
VC
MMP-1


Invasion Assay-U87
Number of cells invaded
70
60
50
40
30
20
10
0
*
P
VC
MMP-1

C


U251MG
% change in [ATP]
800
700
600
500
400
300
200
100
0
day 0
day 1
day 3
day 5
P
VC
MMP-1


U87MG
% change in [ATP]
800
700
600
500
400
300
200
100
0
day 0
day 1
day 3
day 5
P
VC
MMP-1

2.5 Discussion

In this study, we examined the role of MMP-1 in glioma cell invasion and *in-vitro* cell proliferation. It is known that GBMs express MMP-1 (Hodgson et al., 2009; Stojic et al., 2008; McCready et al., 2005; Nakano et al., 1995); however, its specific role in high-grade glioma pathology is not yet determined. Our results demonstrate that MMP-1 promotes the invasive potential of U251MG and U87MG GBM cell lines, and inhibition of its expression in T98G reduces glioma invasion. The ability to affect invasion is not due to a change in cellular *in vitro* proliferation as this is not altered in cells with MMP-1 over-expression or inhibition.

Invasion of brain tumor cells is one of the hallmark challenges that face glioma-targeted therapies. During invasion, proteases released from both host and tumor cells may interact with the extracellular matrix (ECM) molecules to facilitate cell dispersal. Published findings report that gliomas express several proteases including members of the matrix metalloproteinase (MMP) family (Fillmore et al 2001; Bellail et al., 2004; Van Meter et al., 2001, Nuttall et al., 2003). Matrix metalloproteinase-1 is an important contributor to invasion and metastasis in a variety of cancers (Egeblad and Werb, 2002; Vihinen et al., 2002; Ala-Aho and Kahari, 2005; Nikkola et al., 2005; Ghilardi et al., 2001; Brinckerhoff et al., 2000; Wyatt et al., 2005; Behrens et al., 2001; Murray et al.,

1996). Since there is a low abundance of type I collagen in the brain, the role of MMP-1 in brain tumor invasion likely lies in a function distinct from its ability to degrade collagen. In addition to its role of degrading collagen, MMP-1 can also cleave latent precursors of bioactive molecules present in the ECM (Sternlicht and Werb, 2001; Brinckerhoff and Matrisian, 2002). These cleaved activated molecules such as growth factors bind to their receptors leading to signaling cascades that promote tumorigenesis including pro-invasive, proliferative, and anti-apoptotic mechanisms. In this context, we speculate that MMP-1 mediates brain tumor invasion largely by mechanisms other than by degradation of type I collagen.

Recently, it has been shown that MMP-1 directly cleaves protease-activated receptor-1 (PAR-1), a G-protein coupled receptor (GPCR) and promotes invasion in breast cancer (Boire et al., 2006) and melanoma (Blackburn et al., 2009). PAR-1, also a pro-angiogenic receptor, has been proposed to be involved in the invasion and metastasis of cancers of breast, ovarian, pancreas, prostate and melanoma (Boire et al., 2006; Granovsky-Grisaru et al., 2006; Blackburn et al., 2009; Agarwal et al., 2008; Salah et al., 2007) by triggering G-protein signaling mechanisms in the cell that include increased calcium signaling, cell, migration, gene transcription and mitosis. We speculate that such cleavage and activation of PAR-1 by MMP-1 may form one of the mechanisms that lead to increased glioma invasion. PAR-1 activation by MMP-1 not only stimulates pro-invasive GPCR

signaling but also transactivates an important growth factor receptor, EGFR [42-44]. In summary, MMP-1 may perform its pro-invasive functions dually by a) indirectly up regulating pro-invasive factors in ECM and b) cleaving and activating PAR-1 and downstream signaling.

Taken together, these findings suggest that inhibition or down-regulation of MMP-1 may be beneficial in developing a more effective therapeutic approach to improve the treatment of gliomas.

CHAPTER 3

Epidermal Growth Factor induces metalloproteinase-1 (MMP-1) expression and invasion in glioma cell lines via the MAPK pathway.

Monika Anand, Timothy E Van Meter, and Helen L Fillmore

3.1 Abstract

Glioblastoma Multiforme (GBM) is an aggressive cancer with a poor survival rate. A key component that contributes to the poor prognosis is the capacity of glioma cells to invade local brain tissue in a diffuse manner. Among various proteases that aid in the process of invasion, matrix metalloproteinase-1 (MMP-1) has been identified as an important contributory factor in various cancers. Apart from its traditional role in cleaving its primary extra-cellular matrix (ECM) substrates, and like other members of the matrix metalloproteinase family, MMP-1 can activate latent forms of bio-active molecules initiating downstream pro-invasive and pro-oncogenic signaling mechanisms. MMP-1 expression is regulated by several growth factors including epidermal growth factor (EGF). Due to the fact that the epidermal growth factor receptor (EGFR) is aberrantly overexpressed in GBM, we wanted to examine in greater detail the signaling mechanisms by which MMP-1 expression and invasion is driven by EGF in GBM cells. Treatment of T98G cells with EGF resulted in an induction of MMP-1 expression following EGFR activation. Inhibition of EGFR by either pharmacologic or genetic approaches abrogated this induction. Repression of the mitogen activated protein kinase (MAPK) signaling led to the inhibition of EGF-induced MMP-1 whereas the PI3-kinase/AKT signaling was not associated with EGFR-mediated MMP-1 induction. Inhibition of EGFR signaling also led to a decrease in T98G invasion. These data suggests that EGFR mediated MMP-1

regulation is mainly via the MAPK pathway in T98G cells and inhibition of EGFR and MMP-1 results in a decrease in T98G cell invasion.

3.2 Introduction

Glioblastoma Multiforme (GBM) is the most common and malignant primary brain tumor (CBTRUS, Statistical Report, 2010). Despite multimodal treatments involving surgical resection, chemotherapy and radiotherapy, the survival rate is dismal, with a mean survival of approximately 11 months. One major reason for the failure of therapies is the aggressive infiltration and invasive nature of the tumor cells. Mechanisms of invasiveness are therefore important parameters for studies in the identification of potential therapeutic targets for this incurable cancer.

Growth factors, extracellular matrix molecules, and proteases are secreted from both tumor and normal brain cells, and contribute to the highly invasive behavior of GBM cells. Tumor cell invasion involves interactions with and degradation of molecules of the extracellular matrix (ECM) followed during active cell migration. Matrix metalloproteinases (MMPs) are important proteolytic enzymes that have been implicated in the process of invasion in a wide variety of cancers (Egeblad and Werb, 2002).

Amplification, over-expression and mutations in EGFR gene are common events (affecting ~ 65% of cases) in gliomas tumorigenesis, particularly in de-novo GBM (Libermann et al., 1985; Wong et al., 1992; Frederick et al 2000). EGFR is involved in the regulation of cell proliferation, motility and survival of cells and an imbalance in the EGFR-ligand signaling axis can give rise to neoplastic transformation via 1) increased production of EGFR ligands and autocrine regulation, 2) increased expression of EGFR, 3) EGFR mutations that lead to constitutive activation of the receptor, or 4) defects in the normal EGFR down-regulatory processes (Zandi et al., 2007).

Mutations in the extra-cellular domain are particularly frequent in GBM, especially the EGFRvIII mutation, also called as de2-7EGFR or δ2-7EGFR (Zandi et al., 2007). EGFRvIII mutation results from an in-frame deletion of exons 2 to 7 that encode subdomain I and 2/3rd of subdomain II (Wong et al., 1992). The truncated receptor lacks the N-terminal ligand binding area, changing the conformation of the receptor to allow autophosphorylation and constitutive activation (Ekstrand et al., 1994). This activation is enough to induce and sustain oncogenic signaling (Chu et al., 1997) but not sufficient to be recognized by its degradatory proteins (Grandal et al., 2007). Stimulation of EGFR by its ligand EGF leads to receptor homo- or heterodimerization (Olayioye et al., 2000) followed by activation of tyrosine kinase domain of the receptor through trans-tyrosine phosphorylation. The activated receptor kinase phosphorylates the

tyrosine residues on the C-terminal tail of the ErbB receptors. These phosphorylated sites allow the binding of proteins with src-homology (SH2) domains such as Grb2, shc and Nck that act like adaptor proteins that have intracellular docking sites for other proteins such as serine threonine kinases. This relays the signaling activating other serine threonine kinases and transcription factors (Olayioye et al., 2000). Thus, signaling from EGFR leads to an amplification of several signaling pathways that alter protein function and gene transcription conducive for oncogenesis. The best characterized EGFR downstream signaling pathways are the RAS-RAF-MEK-ERK and PI3K-AKT pathways.

Accumulating evidence suggests a correlation between elevated expression of MMPs and glioma progression. Published findings show that MMP-1 (McCready et al., 2005; Stojic et al., 2008), MMP-2 (Gondi et al., 2009), MMP-7 (Vince et al., 1999), MMP-9 (Ezhilarasan et al, 2009; Lakka et al., 2002), MMP-14, MMP-15 (Fillmore et al., 2001; Zhang et al., 2005) and MMP-16 (Xia et al., 2009) are involved in brain tumor biology. Of the matrix metalloproteinase family, MMP-2, MMP-14 and MMP-9 have been the most extensively studied in brain tumors whereas MMP-1 is less well-characterized. We have reported that there is a significant increase in MMP-1 mRNA levels in tumor samples from patients with GBM relative to normal brain (McCready et al., 2005). MMP-1, which is not typically expressed in normal brain, has been shown to be elevated in

gliomas (Hodgson et al., 2009) and correlates with tumor grade and survival time (Stojic et al., 2008).

We wanted to examine if amplified EGFR signaling contributes to pro-invasive MMP-1 protein up-regulation and determine if MMP-1 mediated activity is an important contributor to GBM invasion. Herein, we report that MMP-1 is regulated by EGFR. High expression of MMP-1 is observed after treatment with EGF, and this is dependent on EGFR-driven downstream MAPK signaling. Inhibition of EGFR using the pharmaceutical inhibitor, AG1478 led to the suppression of the EGF induced MMP-1 protein levels. In addition adenoviral mediated transfection of vectors that express dominant negative forms of MEK and EGFR led to the inhibition of EGF induced MMP-1 while cells transfected with adenoviral CA-MEK led to increased MMP-1. Functionally EGF induced MMP-1 expression correlated with an increase in cellular invasion and treatment with AG1478 abrogated this increase. Although not to the same extent, MMP-1 siRNA treated cells also blunted the EGF induced invasion.

Data are expressed as the mean and SEM of at least three independent experiments. Statistical analysis was done using an unpaired Student's *t* test. $P < 0.05$ was considered significant.

3.3 Materials and Methods

Cell Culture

Human GBM cell lines (T98G and U87MG) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM Invitrogen, Carlsbad, CA, USA) supplemented with 4.5 g/l D-glucose, 1% penicillin/streptomycin (Invitrogen) and 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, West Sacramento, CA, USA). Cells were incubated in a humidified incubator at 37°C with 5% CO_2 in Nunclon 75cm^2 culture flasks (Nunc, Germany) and harvested with 0.25% Tryspin-EDTA (Invitrogen). The cells were plated in six-well plates at the density of 200,000 cells/ml for 24h and then pre- treated with inhibitors AG1478 (300nM) and PD184352 (500 nM) (Calbiochem, USA) for 1 hour before adding 20ng/ml EGF (Cell signaling, USA).

Immunoblot

Cell lysates and conditioned media were collected from the GBM cell lines- T98G and U87MG. Prior to extraction of conditioned media and cell lysates, complete serum media was aspirated from six-well plates well and washed with PBS. One ml serum –free media was added to each well and incubated for 24h. Then the conditioned media was aspirated and centrifuged at 1000 X g to remove cell debris. The supernatant was collected and added to Amicon centrifugal filters (Millipore) for concentration of the protein from 3ml to 250 µl. For preparation of cell lysates, the cells were treated with ice cold RIPA buffer (150 mM NaCl, 50mM Tris, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Calbiochem). The lysates were centrifuged at 14000 rpm for 20 min after shearing with 26 gauge needles. Protein concentrations were determined using DC Protein Assay (Bio-Rad, Hercules, CA, USA)

MMP-1 protein level was determined by immunoblot analysis. Proteins isolated from conditioned media (10ug) and cell lysates (30µg) were separated in 4-12% polyacrylamide –bis-Tris gels (Invitrogen, Carlsbad, USA) and transferred to a 0.45µm nitrocellulose membrane (Invitrogen, Carlsbad, USA). After blocking in 5% milk in TBST (50mM Tris base, 150mM NaCl, 0.05% (v/v) Tween 20), the membranes were incubated with mouse monoclonal anti-MMP-1 antibody (R & D Systems, Minneapolis, MN, USA at 1:200 dilution) in the blocking solution. Rabbit polyclonal anti-cyclophilin A antibody (Millipore, Billerica, MA, USA)

was used to quantify any variability in loading onto the polyacrylamide gels. After washing the membranes in TBST, they were incubated with horseradish peroxidase –conjugated secondary antibodies: goat anti rabbit IgG and goat anti mouse IgG, (Rockland Immunochemicals, Gilbertsville PA, USA) both diluted 1:5000 in 5% milk-TBST solution. Blots were visualized with exposure of autoradiographic film using an enhanced chemiluminescent system (ECL, GE Healthcare). The developed films were scanned and densitometry was performed using ImageJ.

Adenoviral Transfections

T98G cells were plated in six-well plates at a density of 200,000 cells per ml in triplicates per condition. The adenovirus-pCMV, CA-AKT, DN-AKT, CA-MEK, DN-MEK (Vector Biolabs, PA, USA) and DN-EGFR (Molecular Biology Core Facility, VCU) were a kind gift from Dr Paul Dent, Virginia Commonwealth University. The CA-AKT has myristoylation signal for membrane targeting and increased activity. The DN-AKT has alanine residues substituted for threonine at position 308 and serine at position 473. The CA-MEK has serine 221 alanine mutation in MAPK kinase and DN-MEK has serine 217/221 glutamine mutation in MAPK kinase. The DN-EGFR lacks 533 C-terminal amino acids as described [21]. The cells were infected with these recombinant adenoviruses at an MOI of 25 for 48 hours. Then the media was aspirated and replaced with serum-free media for overnight before immunoblotting.

MMP-1 siRNA transient transfection

T98G cells were transfected with *Silencer* pre-designed siRNAs against MMP-1: siRNA ID 1157 sense, GGUAUGAUGAAUAUAAACGtt (Ambion, Austin, TX, USA), targeting between 1265 to 1285 bases in the human MMP-1 transcript. The cells were plated in complete media (without antibiotics) in six-well plates at a density of 200,000 cells per well. At 80% confluence, media were removed from the cells and the wells were washed with PBS followed by addition of 800 µl per well of Opti-MEM I reduced serum medium (Invitrogen). Oligofectamine transfection reagent, Opti-MEM I and siRNA or scrambled RNA (at concentrations of 30nM) mixtures were made and incubated at room temperature for 20 min. Then cells were treated with reaction mixtures of scrambled siRNA and predesigned siRNA against MMP-1. The six well plates were kept on a shaker at 37°C for 6 hours. Opti-MEM I supplemented with 30% FBS was added (500 µl per well) for overnight incubation. Complete media were added the next day (500µl per well). At the end of 48 hours, media were replaced with serum-free media for overnight incubation at 37°C. Proteins were extracted from conditioned media and cells after the overnight incubation.

Matrigel Invasion Assays

Invasiveness of GBM cell lines was determined using transwell inserts (Corning Incorporated, USA) with 8µm pores. The inserts were coated with growth factor reduced Matrigel (BD Biosciences, CA, USA) at a concentration of 100µg per

filter diluted in cold serum-free DMEM for 30 minutes at 37°C incubator. 600 µl of 10% serum containing medium was added to the bottom chamber. The cells at a density of $1x10^6$ cells per ml were added to the top chamber and incubated at 37°C for 48 hours. At the end of incubation, media from bottom chamber was removed and trypsin-EDTA was added. The cells from bottom of the filter were scraped and added to trypsinized cells. The cell number was assessed CellTiter-Glo luminescent Cell Viability ATP assay (Promega). A standard curve of known ATP concentrations was generated for each assay. The measurement of number of cells was correlated with the luminescence obtained from serial dilutions of cell-counts from T98G cell line. Percent invasion was calculated as the (luminescence values obtained from analyzing the cells that invaded the matrigel) divided by the (luminescence obtained from cell-count added to the top well) X 100. Data from three separate experiments with five replicates for each condition were analyzed.

Statistical Analysis

Data were evaluated by comparing the means and SEM of replicate experiments. Data are expressed as the mean and SEM of at least three independent experiments. Statistical analysis was done using an unpaired Student's *t* test. $P < 0.05$ was considered significant.

Data are expressed as the mean and SEM of at least three independent experiments. Statistical analysis was done using an unpaired Student's *t* test. $P < 0.05$ was considered significant.

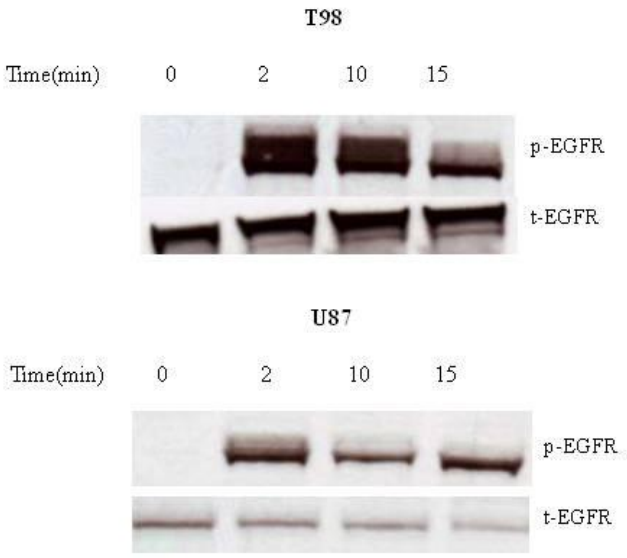
3.4 Results

EGFR mediates regulation of MMP-1

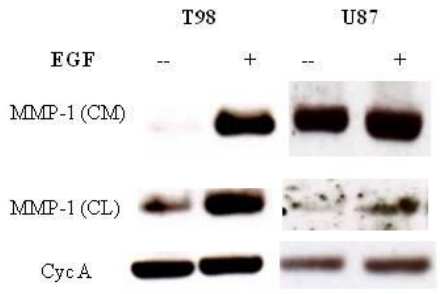
T98G and U87MG glioma cell lines were examined for changes in the expression of MMP-1 protein following EGF treatment. Addition of 20 ng/ml EGF led to phosphorylation and activation of EGFR within 2 minutes and the phosphorylation status was sustained over 15 min and 24 hour time points (**Figure 3.1A**). MMP-1 protein levels were analyzed in T98G and U87MG glioma cell lysates and conditioned media 24 hours following EGF treatment. MMP-1 levels were increased several fold in both cell lines *(T98CL = 9 fold, p <0.05 T98CM= 0.75 fold, p< 0.05 U87CL= 3 fold, p<0.05 U87CM= 6.5fold, p<0.05)* (**Figure 3.1B, 1C**). We next sought to inhibit the activation of EGFR using the small molecule inhibitor, AG1478 in T98G cells. Addition of AG1478 (300 nM) 2h prior to treatment with EGF completely inhibited the activation of EGFR by EGF (**Figure 3.1D**). MMP-1 protein levels were also decreased in samples treated with AG1478 alone. These findings demonstrate that EGFR activation by EGF specifically regulates the expression of MMP-1.

Figure 3.1 Regulation of MMP-1 by EGF Representative immunoblots of T98G and U87MG glioma cell lysates (CL) from **A)** cells treated with EGF (20 ng/ml) for 2, 10 and 15 min show activation of EGFR, **B)** cells treated with EGF (20 ng/ml) for 24h demonstrate induction of MMP-1 in CL and conditioned media (CM). **C)** Densitometric analysis of MMP-1 protein band intensity across three independent experiments, $p^{*}<0.005$ (n=3). **D)** Cells treated with AG1478 (300nM) for 24h show complete inhibition of EGFR activation and MMP-1 protein levels in T98G CL, Densitometric analysis across three independent experiments, $p^{*}<0.05$ (n=3).

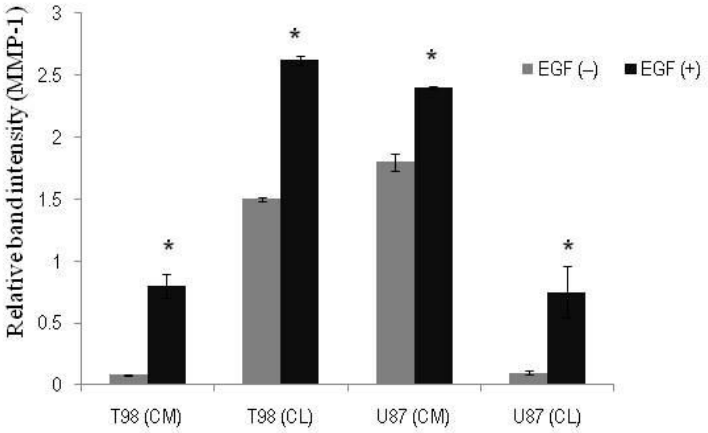
A


T98
Time(min) 0 2 10 15
p-EGFR
t-EGFR
U87
Time(min) 0 2 10 15
p-EGFR
t-EGFR

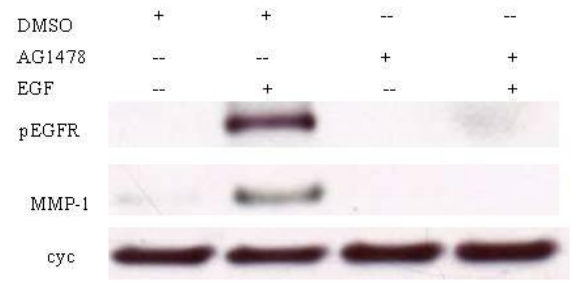
B

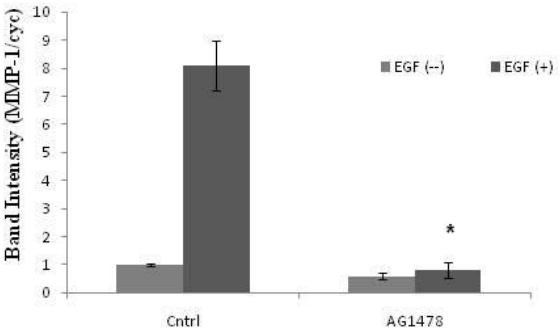

T98
U87
EGF
--
+
--
+
MMP-1 (CM)
MMP-1 (CL)
Cyc A

C


Relative band intensity (MMP-1)
3
2.5
2
1.5
1
0.5
0
EGF (–)
EGF (+)
*
*
*
*
T98 (CM)
T98 (CL)
U87 (CM)
U87 (CL)

D


DMSO + + -- --
AG1478 -- -- + +
EGF -- + -- +
pEGFR
MMP-1
cyc

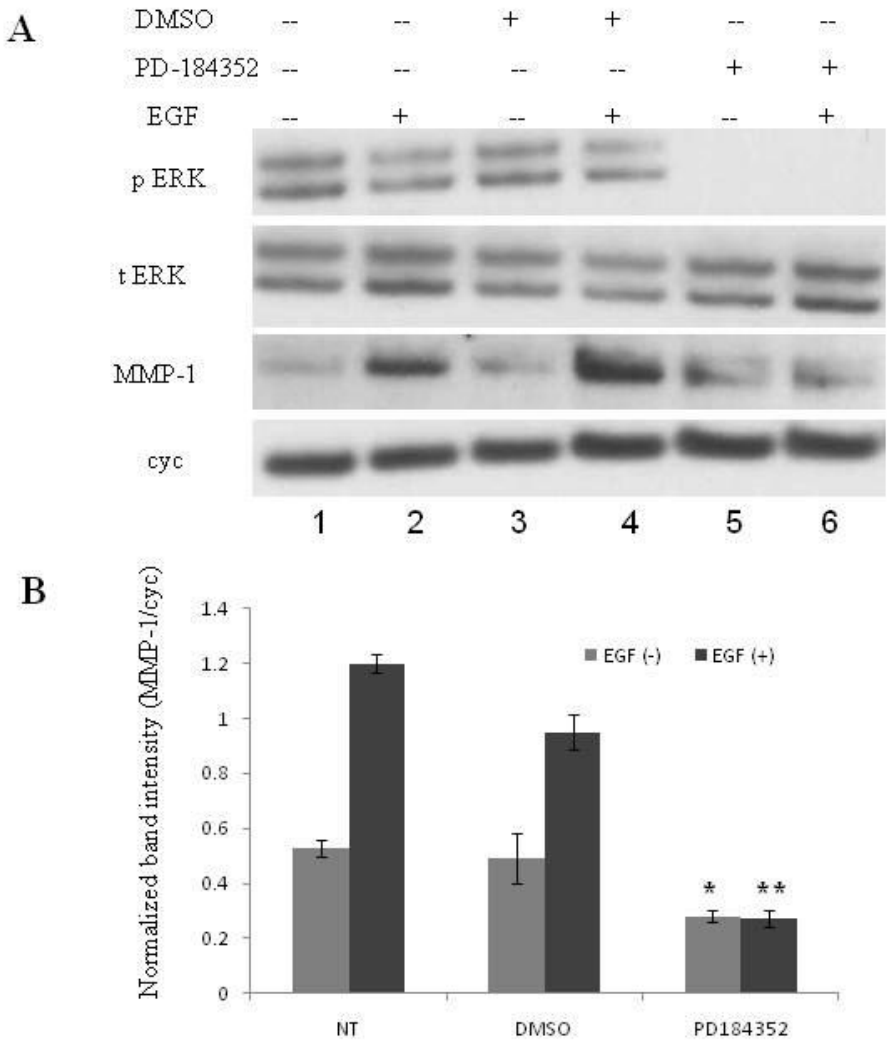

Band Intensity (MMP-1/cyc)
10
9
8
7
6
5
4
3
2
1
0
EGF (--)
EGF (+)
*
Cntrl
AG1478

Involvement of MAPK pathway in the EGF mediated induction of MMP-1

To determine downstream EGFR signaling pathways involved in the increase in MMP-1 expression, T98G cells were first treated with selective MEK1/2 inhibitors U0126 and PD184352. Pre-treatment with 500nM PD184352 for 2 hours before addition of EGF completely inhibited the phosphorylation of ERK (**Figure 3.2A, lanes 5 and 6**). EGF treatment had no effect on phosphorylated or total ERK1/2 levels in this cell line (**Figure 3.2A, lanes 2 and 4**). MMP-1 levels were also examined in T98G cells treated with PD184352 for 24 hours. There was a suppression of MMP-1 induction in the presence of EGF in the cells treated with PD184352 (**Figure 3.2A lanes 5 and 6**) suggesting MMP-1 regulation is effected by the MAPK pathway.

Figure 3.2 Inhibition of MAPK signaling by PD184352 and MMP-1 levels.

A) Representative immunoblot of T98G CL, from cells treated with PD184352 (500nM) for 24h indicating complete inhibition of ERK activation and decreased induction of MMP-1 protein levels in the presence of EGF (CL, cell lysate; ERK, p44/42 Erk1/2 Mitogen activated kinases). **B)** densitometric analysis of MMP-1 band intensity across three separate experiments, $p^{*,\ **}<0.05$ [*****, DMSO (-EGF) Vs PD184352 (-EGF)], [******, DMSO (+EGF) Vs PD184352 (+EGF)] (n=3).

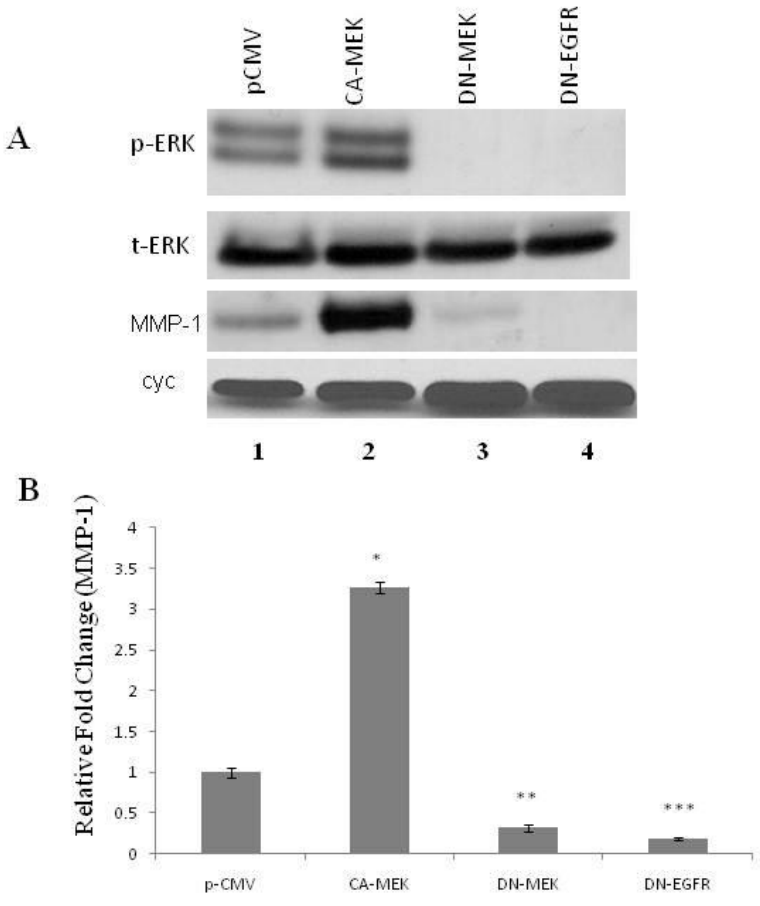

A
DMSO -- -- + + -- --
PD-184352 -- -- -- -- + +
EGF -- + -- + -- +
p ERK
t ERK
MMP-1
cyc
1 2 3 4 5 6
B
Normalized band intensity (MMP-1/cyc)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
EGF (-)
EGF (+)
*
**
NT
DMSO
PD184352

Involvement of MAPK pathway in the EGF mediated induction of MMP-1

To examine more closely the possible involvement of the MAPK signaling pathway in EGF-induction of MMP-1, we used adenoviral constructs targeting MEK and EGFR. Constitutively active (CA) and dominant negative (DN)-MEK and DN-EGFR –adenoviral constructs were transfected in T98G cells and levels of phospho-ERK1/2- and total ERK1/2 and MMP-1 were examined (**Figure 3.3**). The T98G cells transfected with DN-EGFR show decreased levels of p-ERK and MMP-1 as compared to pCMV controls (**Figure 3.3A lanes 1 and 4**). In the presence of DN-MEK, p-ERK and MMP-1 levels were reduced (**Figure3. 3A lane 3**). Conversely CA-MEK increases MMP-1 protein amounts, with a slight increase in the endogenous levels of p-ERK (**Figure 3.3A, lanes 1 and 2**). The DN-MEK and DN-EGFR completely inhibit phosphorylation of ERK suggesting MAPK as an integral component of EGFR downstream signaling.

Figure 3.3 EGFR mediated MAPK signaling influences MMP-1 levels.

A) Representative immunoblot of T98G cells transfected with adenoviral vectors CA-MEK, DN-MEK and DN-EGFR (MOI of 25) show complete inhibition of ERK phosphorylation in cells treated with DN-MEK and DN-EGFR. MMP-1 levels are also decreased in these cell lines. **B)** Densitometric analysis across three independent experiments showing fold change values of MMP-1 protein levels relative to the pCMV control. The CA-MEK resulted in an approximately 3 fold increase in MMP-1 protein levels whereas transfection of DN-MEK decreased MMP-1 levels to 0.3 fold and that of DN-EGFR decreased MMP-1 levels to 0.2 fold relative to the pCMV control (CA-Constitutive active, DN-Dominant negative). Data is representative of three separate experiments, $p^{*, **, ***} < 0.05$ (n=3).

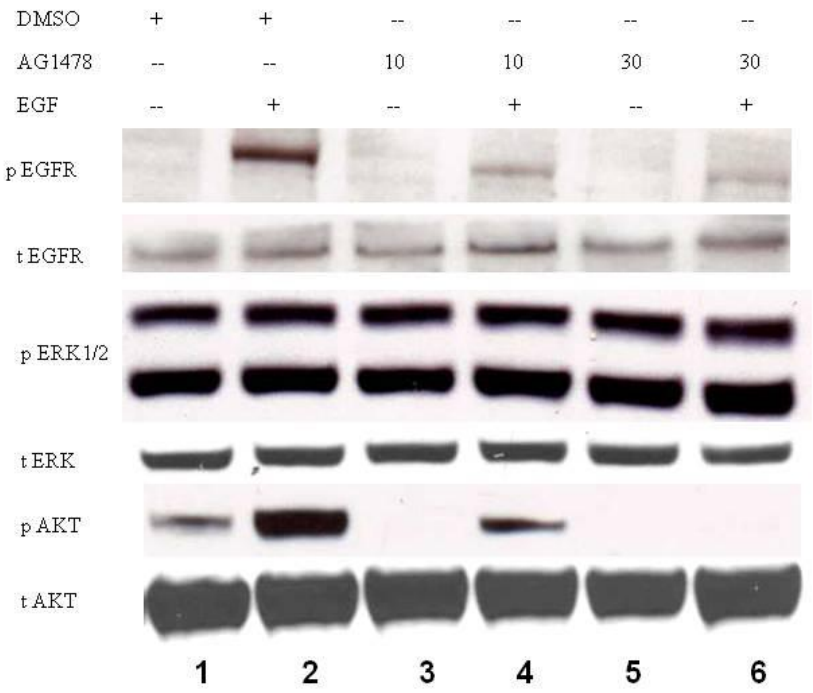

A
pCMV
CA-MEK
DN-MEK
DN-EGFR
p-ERK
t-ERK
MMP-1
cyc
1
2
3
4
B
Relative Fold Change (MMP-1)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
**
***
p-CMV
CA-MEK
DN-MEK
DN-EGFR

Role of PI3-kinase pathway in EGF mediated MMP-1 regulation

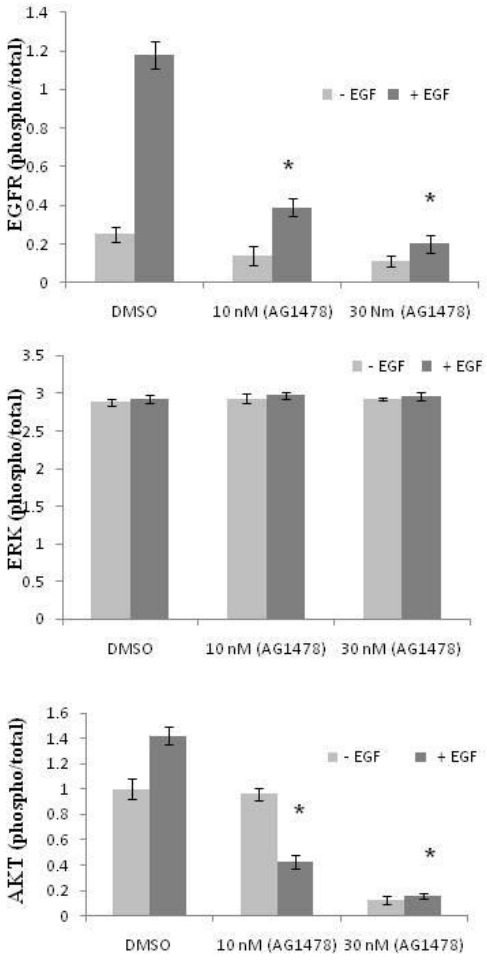
We also wanted to examine the PI3 Kinase pathway and its potential influence on EGF-induced MMP-1 expression. T98G cells were pre treated with two concentrations of AG1478 (10nM and 30nM) for 2 hours and stimulated with EGF (20ng/ml). The activation status of EGFR, AKT and ERK was tested in these treated samples. In the presence of EGF, p-EGFR was inhibited by AG1478 (**Figure 3.4A lanes 1, 3 and 5**). In agreement with Figure 2, EGF had no effect on the p-ERK levels. There was a dose-dependent inhibition of EGF stimulated p-Akt levels with increasing amounts of AG1478 **(Figure 3.4A, lanes 1, 3, and 5**). In addition, basal and EGF-stimulated p-ERK levels were unchanged and the AG1478 treatment did not affect the phosphorylation status of ERK **(Figure 3.4B).** These observations suggest that in addition to the MAPK pathway, EGF-induction of MMP-1 may involve the PI3K pathway.

Figure 3.4 AG1478 mediates its downstream effects via PI3-K signaling **A)** Representative immunoblot of T98G cells treated with two different concentrations of AG1478 (10 and 30 nM) show decreased levels of p-EGFR and p-AKT but no effect on p-ERK. **B)** Densitometric analysis of EGFR, AKT and ERK (phospho- and total) immunoreactivity in the above immunoblots across three separate experiments, p*****<0.05

A


DMSO + + -- -- -- --
AG1478 -- -- 10 10 30 30
EGF -- + -- + -- +
pEGFR
tEGFR
pERK1/2
tERK
pAKT
tAKT
1 2 3 4 5 6

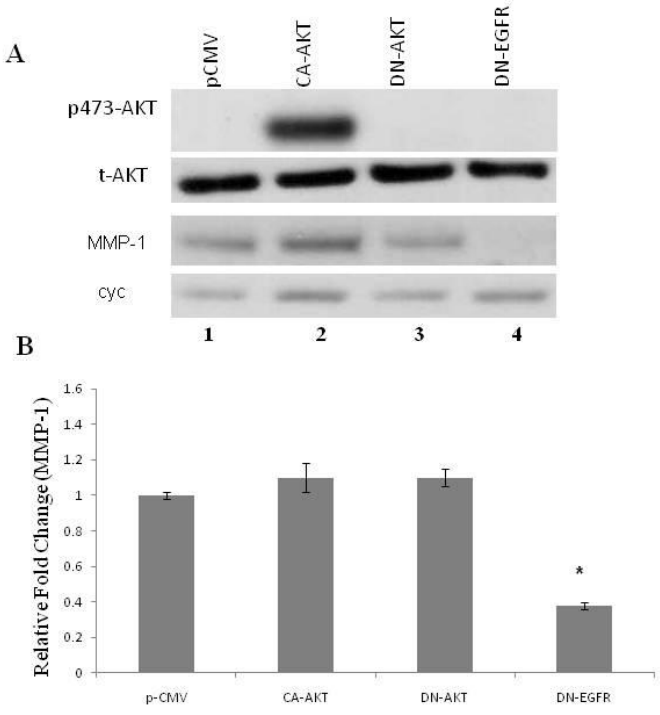
B


EGFR (phospho/total)
- EGF
+ EGF
DMSO
10 nM (AG1478)
30 Nm (AG1478)
ERK (phospho/total)
30 nM (AG1478)
AKT (phospho/total)

Role of PI3-kinase pathway in EGF mediated MMP-1 regulation

To further examine the role of PI3K in EGF induced MMP-1 expression, adenoviral constructs (CA-AKT and DN-AKT) were used to transfect T98G cells. Successful adenoviral transfection was confirmed by immunoblotting to detect phospho-Serine 473-AKT and total-AKT (**Figure 3.5A**). Only the CA-AKT treated T98G cells were shown to have high Serine-473 phosphorylation levels (**Figure 3.5A, lane 2**). Surprisingly, the MMP-1 levels remain same in T98G cells transfected with adenoviral- CA-AKT and DN-AKT plasmids and are similar to the pCMV controls (**Figure 3.5A, lanes 1 to 3**). Consistent with earlier data (**Figure 3.3A lanes 1 and 4**, **3.3B**) T98 cells treated with adenoviral DN-EGFR plasmids demonstrate a decrease in MMP-1 levels to 0.3 fold as compared to the pCMVcontrol (**Figure 3.5A, lanes 1 and 4, Figure 3.5B**).

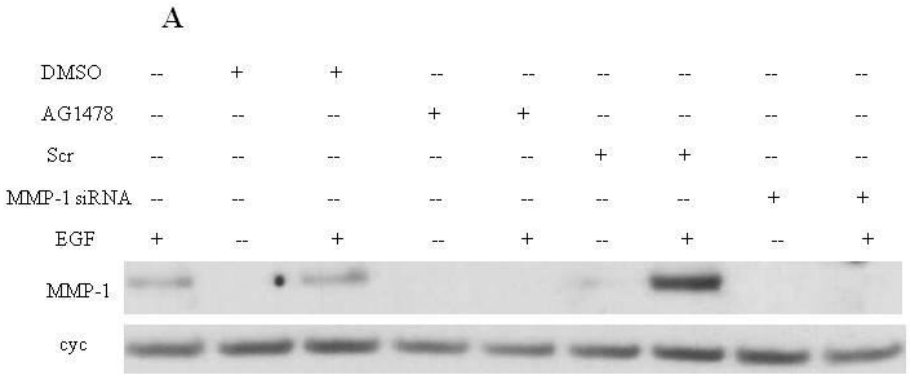
Figure 3.5 PI3-K signaling does not influence MMP-1 levels A) Representative immunolot of T98G cells treated with adenoviral vectors CA-AKT and DN-AKT (MOI of 25) show that CA-AKT led to phosphorylation of AKT at 473 position with no active AKT in the control samples. This increased phosphorylation did not lead to alterations in MMP-1 levels. **B)** Densitometric analysis of the MMP-1 immunoblots across three separate experiments showing fold change values relative to the pCMV control. The DN-EGFR sample group shows a decrease in MMP-1 levels to approx 0.3 fold as compared to the pCMV control group. Data is representative of three independent experiments, p*****<0.05, n=3.


A
pCMV
CA-AKT
DN-AKT
DN-EGFR
p473-AKT
t-AKT
MMP-1
cyc
1
2
3
4
B
Relative Fold Change (MMP-1)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
p-CMV
CA-AKT
DN-AKT
DN-EGFR

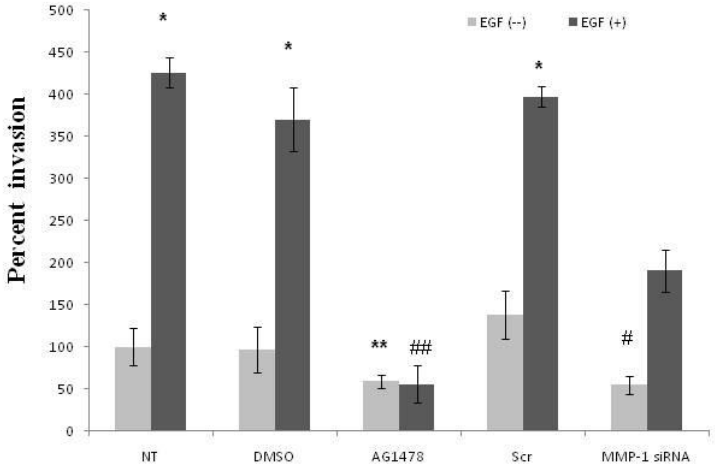
EGF stimulates glioma cels invasion *in vitro*

We next wanted to examine whether MMP-1 upregulation by EGF is an important event in the *in vitro* invasion process in glioma cells. T98G cells were treated with DMSO and AG1478 in the presence and absence of EGF. Inhibition of MMP-1 was performed using transient transfection of MMP-1 siRNA. Treatment with AG1478 alone in T98G cells significantly decreased invasion as compared to the DMSO control. Similarly, MMP-1 siRNA treatment to T98G cells significantly suppressed invasion as compared to the scrambled control ($p < 0.05$) (**Figure 3.6**). The addition of EGF to T98G cells (No treatment, NT and DMSO-treated) led to an increase in invasion ($p<0.05$). The addition of EGF to AG1478 treated T98G cells did not increase invasion suggesting suppression of EGFR-mediated pro-invasive signaling. Interestingly, addition of EGF to MMP-1 siRNA treated T98G cells resulted in an increase in invasion but this increase was not as pronounced as controls. This data suggests that MMP-1 inhibition results in blunting the EGF-mediated increase in invasion and therefore contributes, in part, to EGF-mediated invasion process.

Figure 3.6 EGFR mediated increased invasion involves MMP-1 in T98G cells. Representative immunoblot of T98G cells treated with controls (NT, DMSO), AG1478, scrambled control, MMP-1 siRNA (with and without EGF) show that MMP-1 levels are decreased in AG1478 and MMP-1 siRNA treated cell lysates even in the presence of EGF. EGF stimulation induced MMP-1 protein levels in controls (NT, DMSO and scrambled). **B)** Addition of EGF to T98G cells in controls (NT, DMSO and scrambled) led to a significant increase in matrigel-invasion. In the presence of AG1478, the EGF mediated increased invasion is inhibited. MMP-1 siRNA treated T98G cells show decreased invasion as compared to the controls. In MMP-1 siRNA treated T98G cells, an increase in invasion was observed with addition of EGF. However, the induction of invasion by EGF was suppressed in these MMP-1 siRNA treated cells as compared to the induction of invasion in NT, DMSO and scrambled sample groups. Data is representative of three independent experiments, $p^{*}<0.05$, $p^{\#}$(Scrambled Vs MMP-1 siRNA) <0.05, p^{**}(AG1478 Vs DMSO, No EGF) <0.05, $p^{\#\#}$(AG1478 Vs DMSO, with EGF) <0.05 (n=3).


A
DMSO -- + + -- -- -- -- -- --
AG1478 -- -- -- + + -- -- -- --
Scr -- -- -- -- -- + + -- --
MMP-1 siRNA -- -- -- -- -- -- -- + +
EGF + -- + -- + -- + -- +
MMP-1
cyc

B


Percent invasion
500
450
400
350
300
250
200
150
100
50
0
EGF (--)
EGF (+)
*
*
*
**
##
#
NT
DMSO
AG1478
Scr
MMP-1 siRNA

3.5 Discussion

EGFR expression and mutation is an important hallmark event in many cancers, including glioblastoma (GBM). Particularly, primary GBMs, also known as *de-novo* GBM have been found to have a higher EGFR mutation rate than progressive or secondary GBM (Ohgaki and Kleihues, 2007), and is recognized to be a significant oncogene driving the growth and malignancy these cancers. There is evidence for the involvement of MMP-1 in local diffuse invasion in GBM (Stojic et al., 2008; McCready et al., 2005; Anand M et al, submitted).

In this study, we sought to examine the role of EGFR signaling in the EGF induction of MMP-1. We observed that by perturbing EGFR using EGF stimulation in 2 glioma cell lines, MMP-1 expression was increased. In the same model system, inhibition by AG1478 and EGFR- dominant negative forms, levels of MMP-1 are altered. Subsequently, we explored EGFR downstream MAPK and PI3-K signaling mechanisms. Using a pharmaceutical inhibitor for the MAPK pathway, PD184352, we observed an abrogation in the induction of MMP-1 protein levels in samples treated with the inhibitor and EGF. Neither the addition of EGF nor adenoviral mediated-infection of MEK-CA in T98G cells increased the phosphorylation of ERK above the basal levels of p-ERK. However, the transfection of Ad-EGFR-DN in T98G cells led to the suppression of phosphorylated ERK and abrogated MMP-1 levels suggesting EGFR-mediated

MAPK regulation of MMP-1. In T98G cells infected with an adenoviral-construct MEK-DN, MMP-1 levels were abolished. In addition, infection of T98G cells with adenoviral-MEK-CA led to a robust increase in the MMP-1 protein levels strongly suggesting involvement of MAPK pathway in MMP-1 gene regulation.

AG1478 treatment led to the suppression of p-EGFR and p-AKT levels but did not alter p-ERK levels. It also resulted in decreased MMP-1 levels suggesting that PI3K signaling might also be involved in the EGF-MMP-1 regulation. There was an apparent dose-dependent decrease in phosphorlyated EGFR with AG1478 treatment. In figure 3.4 we show that with two doses of AG1478 (10 and 30nM) there is a modest dose dependent decrease in phosphorylated EGFR. The addition of higher doses of AG1478 (100 and 300 nM) completely inhibited phosphorylation of EGFR (data not shown).

We further explored the PI3-K signaling pathway by infecting T98G cells with Ad-AKT-CA and Ad-AKT-DN. Interestingly, neither Ad-AKT-CA nor Ad-AKT-DN transfection in T98G cells altered MMP-1 levels suggesting that PI3K may not involved in the EGF-induction of MMP-1 protein.

MMP-1 has pro-invasive functions in wide range of malignancies. In order to examine, if EGFR mediated increase in MMP-1 plays a functional role in

T98 cells, we performed invasion assays. There was a significant increase in invasion with EGF treatment and with AG1478 treatment this EGF-driven invasion nis abolished. The MMP-1 siRNA demonstrated a similar effect. When EGF was added to the MMP-1 siRNA treated cells, although not to the same extent as with AG1478, there was a suppression of the EGF-driven invasion. Thus, MMP-1 inhibition is able to blunt the EGF-mediated increase in invasion in T98G cells emphasizing an important contribution to glioma cell *in vitro* invasion by MMP-1.

CHAPTER-4

GENERAL DISCUSSION

In this study, we have shown that MMP-1 is a significant contributor of glioma invasion *in vitro*. Preliminary studies from our laboratory by Dr Jessica McCready showed that MMP-1 mRNA is not detectable in normal brain but its expression is increased in GBM tissue specimens. The MMP-1 protein levels in GBM tissue specimens were examined using immunohistochemistry and confirmed these earlier results. T98G cell lines were used for transient inhibition of MMP-1 as they have high basal expression of MMP-1. Conversely, the U251MG and U87MG cell lines have low basal expression of MMP-1 making them a suitable model for over-expression studies. Inhibition of MMP-1 using siRNA in T98G cell lines showed a decreased invasion while stable over-expression of MMP-1 in U251MG and U87MG GBM cell lines exhibited significantly increased invasion. These MMP-1 over-expressers have the ability to invade growth factor- reduced matrigel barrier (at 100 μg/ filter concentration) within 12 hours as observed under the microscope.

Several MMPs have been implicated in brain tumors; among them, the most extensively studied are MMP-2 and MMP-9 (Kargiotis et al., 2008; Gondi et al., 2009; Ezhilarasan et al., 2009; Lakka et al., 2002, 2005). The studies presented in this dissertation focused on the functional role of MMP-1 in GBM, particularly invasion. MMP-1 contributes to invasion and metastasis in various malignancies including breast, colon, ovary, skin and pancreas (Murray et al., 1996; Kanamori et al., 1999; Ito et al., 1999; Brinckerhoff et al., 2000; Behrens et

al., 2001; Ghilardi et al., 2001; Wyatt et al., 2005; Nikkola et al., 2005; Ala-aho et al., 2005). In most of these cancers, MMP-1 favors tumor progression, growth and invasion by its traditional function of degradation of rigid constituents of ECM. However, brain ECM is devoid of such rigid barriers and it composed of loosely bound hyaluronan, proteoglycans, tenascin-C and thrombospondin. Nonetheless, collagen types IV, V, fibronectin and laminin are present in low amounts along the vessel walls alongside basement membrane and peri-vascular matrix (Bellon et al., 1985). Brain tumors rarely metastasize outside the brain and there is no intravasation in GBM (Bellail et al., 2004). The GBM cells show a pattern of infiltration along the periphery of vessel walls and white matter along with widespread movement within the brain. It is important to note here that despite the presence of very low quantities of known MMP-1 substrates around peri-vascular region, over-expression of MMP-1 increases invasion significantly. It is therefore possible that the increased invasion mediated by over-expression of MMP-1 is not solely due to degradation of ECM. The above findings compelled us to consider additional functions of MMP-1 in ECM dynamics.

MMP-1 alters invasion in GBM by cleaving biologically active molecules

We considered two substrates of MMP-1 that have been shown to be activated: IGFBP2/3 and PAR-1. MMPs function in conjunction with each other and non-traditional substrates for MMPs are continuously being identified. MMP-1 cleaves various bio-active molecules present in the ECM to up-regulate oncogenic

signaling (Sternlicht and Werb, 2001; Brinckerhoff et al., 2002). Literature review lends evidence that insulin-growth factor binding proteins (IGFBPs) present in ECM are cleaved by MMP-1 and MMP-3 (Fowlkes et al., 1994; Rajah et al., 1999). IGFBPs are highly over-expressed and correlate with increased invasion in GBM (Zhang et al., 2002). Silencing of IGFBP-2 in human GBM cells reduced invasion (Fukushima et al., 2007) and its over-expression promotes glioma development and progression (Dunlap et al., 2007). Because of this correlation between MMPs and IGFBPs, we examined the affect of active and functional MMP-1 on IGFBPs (**Appendix E**). In summary, our preliminary *in vitro* data suggests that MMP-1 does not cleave IGFBPs in T98G cells.

Recent studies have shown that MMP-1 directly cleaves a G-protein coupled receptor (GPCR), Protease-activated Receptor (PAR-1), which is also a pro-angiogenic molecule (Boire et al., 2006; Blackburn et al., 2009). Cleavage of PAR-1 leads to activation of downstream G-protein couple signaling that aids in the invasion, metastasis, growth, proliferation and aggressiveness of several malignancies (Granovsky-Grisaru et al., 2006; Salah et al, 2007; Agarwal et al 2008). PAR-1 is expressed extensively in human brain on neurons and astrocytes. Activation of PAR-1 in both astrocytes and human GBM cell lines (U178) increases the intracellular calcium ion concentration suggesting it is equally functionally active in normal and pathological states of brain (Junge et al., 2004).

We tested the presence of PAR-1 in T98G, U251MG and U87MG GBM cell lines via western blot analaysis but were unable to detect PAR-1. This could be due to the low or nil expression of PAR-1 in *in vitro* conditions in these cell lines and a more sensitive method such as ELISA is needed to detect the receptor. It would be interesting to examine the PAR-1 cleavage and activation of downstream GPCR signaling in the U251MG cells stably over-expressing MMP-1. The high levels of MMP-1 protein expressed by U251-MMP-1OE clones should be activated to achieve a functional and active form of MMP-1. This can be obtained by treating the conditioned media from these cells with APMA followed by dialysis for removal of the organo-mercurial compound. The resulting conditioned media can be used to treat the cells for examining the PAR-1 cleavage. The PAR- cleavage can be determined by measuring the PAR-1 cleaved fragment using ELISA and intracellular concentration of calcium ions.

The *in-vitro* invasion assays performed with MMP-1 over-expressing U251MG and U87MG cell lines showed an increase in invasion without altering the cell-proliferation rate. In the same lines, siRNA inhibition of MMP-1 in T98G cell lines showed a decreased invasion without affecting the cell viability and proliferation rate as measured by cell proliferation assays. Thus, MMP-1 may affect on the cell morphology which changes during the physical movement of the cell. In experiments where recombinant active MMP-1 was added to the cells during the invasion assay, we observe no difference in invasion as compared to

the controls. Based on these findings, one would also believe that MMP-1 is not affecting the growth or proliferation of the cells *in-vitro*. However, in a parallel *in-vivo* study performed by my colleague, Dr Nicholas A Pullen, we observed that these MMP-1 over-expressing U251MG cell lines show significant increase in tumor growth and volume. Moreover, the T98G GBM cell lines stably transfected with lentiviral MMP-1 shRNA show a stark decrease in tumor volume (Pullen et al., 2010–submitted). These results emphasize the importance of the tumor microenvironment and its role in mediating the effects of MMP-1.

A majority of GBM patients have amplification, over-expression and/ or mutation in the EGFR making this particular receptor an attractive target for therapeutics. EGFR is known to up-regulate various oncogenes that are implicated in cell proliferation, invasion, metastasis and tumor growth.

In this study we demonstrated the induction of MMP-1 by EGF. EGF, at concentration of 20ng/ml for 24h show an induction of MMP-1 in T98G and U87MG cell lines. Using a pharmaceutic inhibitor of EGFR, AG1478 at a dose of 300 nM, we observed a complete inhibition of EGFR activation. After the treatment with AG1478 (300 nM) in T98G cell lines for 24 hour time period, we observed that MMP-1 levels are completely abolished in the AG1478 treated samples as compared to the controls. This data confirms that EGFR is playing an important role in the regulation of MMP-1 expression.

To understand the downstream signaling mechanisms that are mediating the EGF-MMP-1 regulation, we used pharmaceutic inhibitors to two of the most important and extensively studied pathways- MAPK and PI3-K/AKT. For MAPK inhibition, we used the U0126 inhibitor at a dose of 30 μM. Inhibition of PI3K-AKT pathway was performed using LY294002 (5 μM). We observed that with U0126, phosphorylation of ERK (p-ERK) is inhibited and over a 24 hour time period, the MMP-1 levels are also abrogated. These experiments were performed in the presence and absence of EGF (20 ng/ml). We also notice that addition of EGF did not increase p-ERK levels above the existing high basal levels. Treatment of T98G cells with LY294002 did not prove to be conclusive because the cells did not survive at the period of 24 h. A dose dependent inhibition experiment was performed using the drug that shows complete inhibition of p-AKT starting at 5 μM and almost complete inhibition at 10 μM. Nonetheless, the cells did not tolerate lower doses of the drug over a 24 h time period. To perform MAPK inhibition at a more physiologically relevant dose, we also used another inhibitor, PD184352 at 500 nM. This inhibitor treatment resulted in same results as U0126 lending credence to the involvement of MAPK in MMP-1 regulation.

An important observation was made in T98G cells treated with both agonist (EGF) and antagonist (AG1478) of EGFR. In these cells, EGF led to an activation of EGFR and AKT. Inhibition of EGFR receptor activation also

resulted in decreased p-AKT levels. However, the levels of p-ERK were entirely unaffected with EGF and AG1478. At a 10 nM dose of AG1478, the extent of inhibition on AKT is more pronounced than on its primary target, EGFR. This may suggest that AKT pathway could be very sensitive to inhibition of EGFR; a small inhibition in the receptor results in a marked decrease in the activation of AKT.

A more specific approach to inhibit the signaling pathways was adopted using adenoviral vectors that target the effector proteins of the pathways. These adenoviral vectors were a kind gift from Dr Paul Dent laboratory, Department of Biochemistry, Virginia Commonwealth University. The recombinant adenoviral vectors were EGFR-dominant negative (DN), AKT-constitutive active (CA), AKT-DN, MEK-CA and MEK-DN. We observed that infection with Ad-EGFR-DN decreased the MMP-1 protein levels substantiating our earlier results with the pharmaceutic inhibitor, AG1478. Ad-MEK-DN remarkably decreased the MMP-1 levels and also inhibited the activation of ERK. Surprisingly, Ad-EGFR-DN also resulted in the inhibition of ERK activation suggesting MAPK is indeed downstream to EGFR signaling. Ad-AKT-CA infection show increased phosphorylation and activation of AKT at position serine -473. However, there was no change in the levels of MMP-1 with Ad-AKT-CA and Ad-AKT-DN. This data strongly highlights the involvement of MAPK pathway in EGFR mediated regulation of MMP-1.

The fact that we could not detect a change in p-ERK levels following addition of EGF could be due to high basal p-ERK expression in T98 cells. . The increase in p-ERK levels with EGF may not be detectable over this high-basal level. With addition of adenoviral dominant negative-EGFR, we observe complete inhibition of the ERK phosphorylation, however this inhibition was not observed with AG1478, a pharmaceutic inhibitor. AG1478 is a reversible inhibitor that binds to the ATP-binding pocket of the activated receptor. Our earlier results with AG1478 pointed to inhibition of AKT at much lower doses than it inhibits its specific receptor, EGFR. This suggests that AKT pathway is more sensitive to AG1478 treatment whereas inhibition of MAPK pathway requires more permanent and robust approach, more so, as the basal levels of pERK are high.

We found that in Ad-MEK-CA transfections, there is a slight increase in the phosphorylation of ERK. We had expected a more robust increase of pERK due to the large increase in MMP-1 exprssion. There is a possibility of cross-talk mechanisms between MEK1/2 and JNK. Published findings hint that ERK may not the only target for MEK1/2 (Adler et al., 2005). In U251 GBM cell lines, hyperphosphorylated MEK1/2 can phosphorylate JNK which further activates p38 MAPKs (Adler et al., 2005). These proteins increase the pool of AP-1 transcription factors in the cell and can lead to increases in MMP-1 transcription.

We also explored the role of another important signaling pathway downstream to EGFR in the regulation of MMP-1- the JAK/STAT pathway. In T24 bladder cancer cells, STAT3 activation was found to be important and necessary for MMP-1 induction by EGFR (Itoh et al., 2006). We wanted to determine if such a mechanism may also be part of GBM cells. To investigate the role of JAK/STAT pathway, we used siRNA against STAT3 in T98G cell lines and examined MMP-1 levels **(Appendix F).**

We had expected that STAT3 siRNA would decrease the levels of MMP-1. But, we observed completely contrary to what we had anticipated. The STAT3 siRNA treated T98G cells show an induction of MMP-1 as compared to the scrambled and transfection regeant control. To determine if other signaling pathways become activated in the event of STAT3 depletion as a compensatory mechanism, we examined the MAPK pathway activation by analyzing p-ERK levels. We observed that pERK levels did not change in the STAT3 siRNA T98G cell lines. In the absence of any stimulatory signal (such as EGF), we could not detect and discern any differences in pAKT levels, although the total AKT levels remain constant. Recent studies show that STAT3 has a tumor suppressive function in the GBM cells. Interleukin-8 (IL-8) is a direct repressed target gene for STAT3 (de la Iglesia et al., 2008). This repression is released during siRNA inhibition of STAT3 thereby allowing IL-8 to mediate its downstream signaling that may up-regulate MMP-1 expression.

We have seen that in GBM cell lines, EGFR is regulating MMP-1 expression preferentially by the MAPK pathway. We next sought to determine if EGFR mediated increased MMP-1 has a functional role of invasion. The T98G cells were treated with controls, AG1478 and MMP-1 siRNA and allowed to invade in a matrigel-based invasion assay in the presence and absence of EGF. We show here that MMP-1 siRNA significantly decreased invasion as compared to the controls corroborating our earlier data. In controls, the EGF treatment led to a significantly increased invasion but in the presence of AG1478, the cells drastically reduced this EGF-mediated increased invasion. Addition of EGF to MMP-1 siRNA treated T98G cells resulted in a blunting of the EGF-driven increase in glioma invasion. MMPs are regarded as main players in the invasion process and as such other MMPs may be regulated by EGF and EGFR. Previous published reports by our laboratory (Van Meter et al., 2004) report the induction of MT1-MMP by EGF stimulation.

In summary, we have shown that MMP-1 plays a role in GBM invasion *in vitro*. The increased expression of MMP-1 could be attributed to the complex milieu of tumor microenvironment that is teeming with growth factors and oncogenic stimuli. In our studies, we found that EGFR is regulating MMP-1 expression by MAPK pathway in T98G GBM cell lines. The finding by others that MMP-1 can cleave PAR-1 is an important development in MMP-1 biology. One proposed hypothesis is that once activated MMP-1 cleaves PAR-1, not only

the GPCR, but other RTKs get activated by transactivation mechanisms. There are unpublished reports that hint that EGFR can be shed by MMPs (personal communication). The activation of EGFR and GPCR signaling pathways up-regulate various oncogenic and invasive genes including MMP-1 thereby creating a feedback loop (**Figure 4.1**). With the aid of tumor micro-environment, cancer cells must maintain such feedlack loop constitutively for survival and growth.

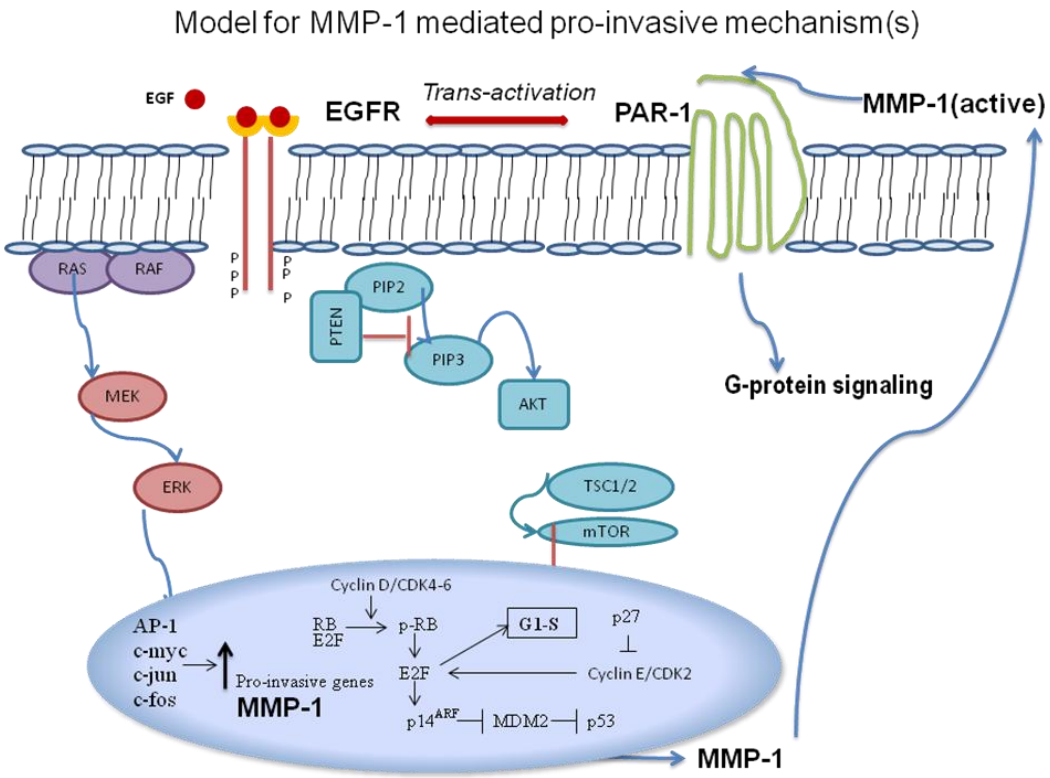

Model for MMP-1 mediated pro-invasive mechanism(s)
EGF
EGFR
Trans-activation
PAR-1
MMP-1(active)
RAS
RAF
PIP2
PTEN
PIP3
AKT
G-protein signaling
MEK
ERK
TSC1/2
mTOR
Cyclin D/CDK4-6
AP-1
c-myc
c-jun
c-fos
RB
E2F
p-RB
G1-S
p27
E2F
Cyclin E/CDK2
Pro-invasive genes
MMP-1
p14ARF
MDM2
p53
MMP-1

Figure 4.1

LITERATURE CITED

Adler V, Qu Y, Smith SJ, Izotova L, Pestka S, Kung HS, Lin M, Friedman FK, Chie L, Chung D, Boutjdir M, Pincus MR. 2005. Functional interactions of Raf and MEK with Jun-N-Terminal Kinase (JNK) result in a positive feedback loop on the oncogenic Ras signaling pathway. Biochemistry 44(32):10784-10795

Agarwal A, Covic L, Sevigny LM, Kaneider NC, Lazarides K, Azabdaftari G, Sharifi S, Kuliopulos A. 2008. Targeting a metalloprotease-PAR1 signaling system with cell-penetrating pepducins inhibits angiogenesis, ascites, and progression of ovarian cancer. Mol Cancer Ther. 7(9):2746-2757.

Agrawal S, Anderson P, Durbeej M, van Rooijen N, Ivars F, Opdenakker G, Sorokin LM. 2006. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. J Exp Med. 203(4):1007–1019.

Akiyama Y, Jung S, Salhia B, Lee S, Hubbard S, Taylor M, Mainprize T, Akaishi K, van Furth W, Rutka JT. 2001. Hyaluronate receptors mediating glioma cell migration and proliferation. J Neurooncol. 53(2):115–127.

Ala-aho R, Kähäri VM. 2005. Collagenases in cancer. Biochimie. 87(3-4):273-286

Amalinei C, Caruntu ID, Giusca SE, Simona EG, Balan RA. 2010. Matrix metalloproteinases involvement in pathologic conditions. Rom J Morphol Embryol. 51(2):215-228.

Amar AP, DeArmond SJ, Spencer DR, Coopersmith PF, Ramos DM, Rosenblum ML. 1994. Development of an in vitro extracellular matrix assay for studies of brain tumor cell invasion. J Neurooncol. 20(1):1–15.

Ancha HR, Kurella RR, Stewart CA, Damera G, Ceresa BP, Harty RF. 2007. Histamine stimulation of MMP-1(collagenase-1) secretion and gene expression in gastric epithelial cells: role of EGFR transactivation and the MAP kinase pathway. Int J Biochem Cell Biol. 39(11):2143-52.

Arora P, Cuevas BD, Russo A, Johnson GL, Trejo J. 2008. Persistent transactivation of EGFR and ErbB2/HER2 by protease-activated receptor-1 promotes breast carcinoma cell invasion. Oncogene. 27(32):4434-4445.

Atkinson JM, Gilbertson RJ, Rich JN. 2009. Brain cancer stem cells as targets of novel therapies. In: Van Meir EG, ed. CNS Cancer: Models, Markers, Prognostic Factors, Targets and Therapeutic Approaches. New York: Humana Press (Springer). 1st ed. 1157–1175.

Aziz F, Kuivaniemi H. 2007. Role of matrix metalloproteinase inhibitors in preventing abdominal aortic aneurysm. Ann Vasc Surg. 21(3): 392–401.

Bailey P, Cushing H. 1925. Microchemical color reactions as an aid to the identification and classification of brain tumors. Proc Natl Acad Sci. 11(1):82-84.

Balbin M, Fueyo A, Tester AM, Pendas AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD, Lopez–Otin C. 2003. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet. 35(3):252–257

Barker FG 2nd, Simmons ML, Chang SM, Prados MD, Larson DA, Sneed PK, Wara WM, Berger MS, Chen P, Israel MA, Aldape KD. 2001. EGFR overexpression and radiation response in glioblastoma multiforme. Int J Radiat Oncol Biol Phys. 51(2): 410–418.

Barker M, Hoshino T, Gurcay O, Wilson CB, Nielsen SL, Downie R, Eliason J. 1973. Development of an animal brain tumor model and its response to therapy with 1,3–bis(2–chloroethyl)–a–nitrosourea. Cancer Res. 33(5):976–986.

Bauer EA, Eisen AZ, Jeffrey JJ. 1970. Immunologic relationship of a purified human skin collagenase to other human and animal collagenases. Biochim Biophys Acta. 206(1):152–160

Bello MJ, de Campos JM, Kusak ME, Vaquero J, Sarasa JL, Pestana A, Rey JA. 1994. Molecular analysis of genomic abnormalities in human gliomas. Cancer Genet Cytogenet. 73(2):122–129.

Bellail AC, Hunter SB, Brat DJ, Tan C, Van Meir EG. 2004. Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. Int J Biochem Cell Biol. 36(6):1046–1069.

Benbow U, Brinckerhoff CE. 1997. The AP–1 site and MMP gene regulation: what is all the fuss about? Matrix Biol. 15(8-9):519–526.

Behrens P, Rothe M, Florin A, Wellmann A, Wernert N. 2001. Invasive properties of serous human epithelial ovarian tumors are related to Ets-1, MMP-1 and MMP-9 expression. Int J Mol Med. 8(2):149-54.

Biernat W, Kleihues P, Yonekawa Y, Ohgaki H. 1997. Amplification and overexpression of MDM2 in primary (de novo) glioblastomas. J Neuropathol Exp Neurol. 56(2):180–185.

Blackburn JS, Liu I, Coon CI, Brinckerhoff CE. 2009. A matrix metalloproteinase-1/protease activated receptor-1 signaling axis promotes melanoma invasion and metastasis. Oncogene. 28(48):4237-4248.

Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A. 2005. PAR1 is a matrix metalloproteinase-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. Cell 120(3):303-313.

Brinckerhoff CE, Matrisian LM. 2002. Matrix metalloproteinases: a tail of a frog that became a prince. Nat Rev Mol Cell Biol. 3(3):207-214.

Brinckerhoff CE, Rutter JL, Benbow U. 2000. Interstitial collagenases as markers of tumor progression. Clin Cancer Res. 6(12):4823-4830.

Burger P, Scheithauer BW. 1994. Tumors of the central nervous system. Washington, DC:Armed Forces Institute of Pathology.

Campbell K, Gotz M. 2002. Radial glia: multi-purpose cells for vertebrate development. Trends Neurosci. 25(5): 235-238.

Carballo E, Lai WS, Blackshear PJ. 2000. Evidence that tristetraprolin is a physiological regulator of granulocyte–macrophage colony-stimulating factor messenger RNA deadenylation and stability. Blood. 95(6):1891–1899.

Carter S, Auer KL, Reardon DB, Birrer M, Fisher PB, Schmidt-Ullrich R, Valerie K, Mikkelsen R, Dent P. 1998. Inhibition of mitogen activated protein kinase cascade potentiates cell killing by low dose ionizing radiation in A431 human squamous carcinoma cells. Oncogene. 16(21):2787–2796.

CBTRUS 2010 Statistical Report

Chakravarti A, Chakladar A, Delaney MA, Latham DE, Loeffler JS. 2002. The epidermal growth factor receptor pathway mediates resistance to sequential administration of radiation and chemotherapy in primary human glioblastoma cells in a RAS-dependent manner. Cancer Res 62(15): 4307–4315.

Chang SM, Parney IF, Huang W, Anderson FA Jr, Asher AL, Bernstein M, Lillehei KO, Brem H, Berger MS, Laws ER; Glioma Outcomes Project Investigators. 2005. Patterns of care for adults with newly diagnosed malignant glioma. JAMA. 293(5):557–564.

Choe G, Park JK, Jouben–Steele L, Kremen TJ, Liau LM, Vinters HV, Cloughesy TF, Mischel PS. 2002. Active matrix metalloproteinase 9 expression is associated with primary glioblastoma subtype. Clin Cancer Res. 8(9):2894–2901.

Chu CT, Everiss KD, Wikstrand CJ, Batra SK, Kung HJ, Bigner DD. 1997. Receptor dimerization is not a factor in the signalling activity of a transforming variant epidermal growth factor receptor (EGFR vIII). Biochem. J 324 (Pt 3): 855-861.

Colnot C, Thompson Z, Miclau T, Werb Z, Helms JA. 2003. Altered fracture repair in the absence of MMP9. Development. 130(17):4123–4133.

Conant K, Haughey N, Nath A, St. Hillaire C, Gary DS, Pardo CA, Wahl LM, Bilak M, Milward E, Mattson MP. 2002. Matrix metalloproteinase-1 activates a pertussis toxin-sensitive signaling pathway that stimulates the release of matrix metalloproteinase-9. J Neurochem. 82(4):885-893.

Daub H, Weiss FU, Wallasch C, Ullrich A. 1996. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature. 379(6565):557-560.

Davis GE. 1992. Affinity of integrins for damaged extracellular matrix: $\alpha_v\beta_3$ binds to denatured collagen type I through RGD sites. Biochem Biophys Res Commun. 182(3):1025–1031.

Davis F, McCarthy B. 2001. Current epidemiological trends and surveillance issues in brain tumors. Expert Rev Anticancer Ther. 1 (3):395–401.

Davis FG, McCarthy BJ, Freels S, Kupelian V, Bondy ML. 1999. The conditional probability of survival of patients with primary malignant brain tumors: surveillance, epidemiology, and end results (SEER) data. Cancer 85(2): 485–491.

De la Iglesia N, Konopka G, Lim KL, Nutt CL, Bromberg JF, Frank DA, Mischel PS, Louis DN, Bonni A. 2008. Deregulation of a STAT3- interleukin 8 signaling pathway promotes human glioblastoma cell proliferation and invasiveness. J Neurosci. 28(23): 5870-5878.

Dent P, Yacoub A, Contessa J, Caron R, Amorino G, Valerie K, Hagan MP, Grant S, Schmidt-Ullrich R. 2003. Stress and radiation-induced activation of multiple intracellular signaling pathways. Radiat Res 159(3): 283-300.

Deryugina EI, Quigley JP. 2006. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev. 25(1):9–34.

Du B, Wang P, Guo X, Du B. 1999. Expression of membrane type 1–matrix metalloproteinase in laryngeal carcinoma. Pathol Oncol Res. 5(3):214–217

Dunlap SM, Celestino J, Wang H, Jiang R, Holland EC, Fuller GN, Zhang W. 2007. Insulin-like growth factor binding protein 2 promotes glioma development and progression. Proc Natl Acad Sci USA 104(28):11736-41.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2(3):161–174.

Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, Collins VP, James CD. 1994. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene 9(8):2313–2320.

Endo K, Takino T, Miyamori H, Kinsen H, Yoshizaki T, Furukawa M, Sato H. 2003. Cleavage of syndecan–1 by membrane type matrix metalloproteinase-1 stimulates cell migration. J Biol Chem. 278(42):40764–40770.

Esparza J, Kruse M, Lee J, Michaud M, Madri JA. 2004. MMP–2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP–9 expression and activity. FASEB J. 18(14):1682–1691.

Esteller M, Garcia–Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG. 2000. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med. 343(19):1350–4

Etoh T, Inoue H, Yoshikawa Y, Bernard GF, Kitano S, Mori M. 2000. Increased expression of collagenase-3 (MMP–13) and MT1–MMP in eosophageal cancer is related to cancer aggressiveness. Gut. 47(1):50–56.

Ezhilarasan R, Jadhav U, Mohanam I, Rao JS, Gujrati M, Mohanam S. 2009. The hemopexin domain of MMP-9 inhibits angiogenesis and retards the growth of intracranial glioblastoma xenograft in nude mice. Int J Cancer. 124(2):306-315.

Ferguson S, Lesniak MS. 2005. Percival Bailey and the classification of brain tumors. Neurosurg Focus. 18 (4):e7.

Fillmore HL, Shurm J, Furqueron P, Prabhu SS, Gillies GT, Broaddus WC. 1999. An in-vivo rat model for visualizing glioma tumor cell invasion using stable persistent expression of green fluorescent protein. Cancer Lett 141(1-2):9-19.

Fillmore HL, VanMeter TE, Broaddus WC. 2001. Membrane-type matrix metalloproteinases (MTMMPs): expression and function during glioma invasion. J Neurooncol. 53(2):187-202.

Folgueras AR, Pendas AM, Sanchez LM, Lopez–Otin C. 2004. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. Int J Dev Biol. 48(5–6):411–424.

Fowlkes JL, Enghild JJ, Suzuki K, Nagase H. 1994. Matrix metalloproteinases degrade insulin–like growth factor binding protein–3 in dermal fibroblast cultures. J Biol Chem. 269(41):25742–25746

Frederick L, Wang XY, Eley G, James CD. 2000. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. Cancer Research 60(5):1383–1387.

Frisch SM, Ruoslahti E. 1997. Integrins and anoikis. Curr Opin Cell Biol. 9(5):701–706.

Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P, Ohgaki H. 2000. Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. Lab Invest. 80(1):65–72

Fukushima T, Tezuka T, Shimomura T, Nakano S, Kataoka H. 2007. Silencing of insulin-like growth factor-binding protein-2 in human glioblastoma cells reduced both invasiveness and expression of progression-associated gene CD24. J Biol Chem. 282(25):18634-18644.

Geho DH, Bandle RW, Clair T, Liotta LA. 2005. Physiological Mechanisms of tumor-cell invasion and migration. Physiology (Bethesda) 20:194–200.

Gersh I, Catchpole HR. 1949. The organization of ground substance and basement membrane and its significance in tissue injury, disease and growth. Am J Anat. 85(3):457–507.

Geoerger B, Gaspar N, Opolon P, Morizet J, Devanz P, Lecluse Y, Valent A, Lacroix L, Grill J, Vassal G. 2008. EGFR tyrosine kinase inhibition radiosensitizes and induces apoptosis in malignant glioma and childhood ependymoma xenografts. Int. J. Cancer. 123(1):209–216

Ghilardi G, Biondi ML, Mangoni J, Leviti S, DeMonti M, Guagnellini E, Scorza R. 2001. Matrix metalloproteinase–1 promoter polymorphism 1G/2G is correlated with colorectal cancer invasiveness. Clin Cancer Res. 7(8):2344–2346.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science. 285(5430):1028–1032.

Giese A, Bjerkvig R, Berens ME, Westphal M. 2003. Cost of migration: invasion of malignant gliomas and implications for treatment. J Clin Oncol. 21(8):1624-1636

Godard S, Getz G, Delorenzi M, Farmer P, Kobayashi H, Desbaillets I, Nozaki M, Diserens AC, Hamou MF, Dietrich PY, Regli L, Janzer RC, Bucher P, Stupp R, de Tribolet N, Domany E, Hegi ME. 2003. Classification of human astrocytic gliomas on the basis of gene expression: a correlated group of genes with angiogenic activity emerges as a strong predictor of subtypes. Cancer Res. 63(20):6613–6625.

Goldberg GI, Wilhelm SM, Kronberger A, Bauer EA, Grant GA, Eisen AZ. 1986. Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation–induced rat protein. J Biol Chem 261(14): 6600–6605

Gondi CS, Dinh DH, Klopfenstein JD, Gujrati M, Rao JS. 2009. MMP-2 downregulation mediates differential regulation of cell death via ErbB-2 in glioma xenografts. Int J Oncol. 35(2):257-263

Grandal MV, Zandi R, Pedersen MW, Willumsen BM, van Deurs BV, Poulsen HS. 2007. EGFR vIII escapes down-regulation due to impaired internalization and sorting to lysosomes. Carcinogenesis 28(7):1408-1417.

Granovsky-Grisaru S, Zaidoun S, Grisaru D, Yekel Y, Prus D, Beller U, Bar-Shavit R. 2006. The pattern of Protease Activated Receptor 1 (PAR1) expression in endometrial carcinoma. Gynecol Oncol. 103(3):802-806

Gross J, Lapiere CM. 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci USA. 15(48):1014–1022.

Grzmil M and Hemmings BA. 2010. Deregulated signaling networks in human brain tumours. Biochim et Biophys Acta 1804(3): 476-483.

Guo W, Giancotti FG. 2004. Integrin signalling during tumour progression. Nat Rev Mol Cell Biol. 5(10):816–826.

Gutin PH, Wilson CB, Kumar ARV, Boldrey EB, Levin V, Powell M, Enot KJ. 1975. Phase II study of procarbazine, CCNU, and vincristine combination chemotherapy in the treatment of malignant brain tumors. Cancer. 35(5):1398–1404.

Hadjipanayis CG, Van Meir EG. 2009. Brain cancer propagating cells: biology, genetics and targeted therapies. Trends Mol Med. 15(11):519–530.

Hamano Y, Zeisberg M, Sugimoto H, Lively JC, Maeshima Y, Yang C, Hynes RO, Werb Z, Sudhakar A, Kalluri R. 2003. Physiological levels of tumstatin, a fragment of collagen IV α3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via αV β3 integrin. Cancer Cell. 3(6):589–601.

Hanahan D and Weinberg RA. 2000. The hallmarks of cancer. Cell. 100(1):57-70.

Harpold H, EC J, Swanson K. 2007. The evolution of mathematical modeling of glioma proliferation and invasion. J Neuropathol. Exp. Neurol. 66 (1):1–9

Haro H, Crawford HC, Fingleton B, MacDougall JR, Shinomiya K, Spengler DM, Matrisian LM. 2000. Matrix metalloproteinase-3-dependent generation of a macrophage chemoattractant in a model of herniated disc resorption. J. Clin. Invest. 105(2):133–141.

Hegi ME, Diserens AC, Godard S, et al. 2004. Clinical trial substantiates the predictive value of O–6–methylguanine–DNA methyltransferase promoter methylation in

glioblastoma patients treated with temozolomide. Clin Cancer Res. 10(6):1871-1874.

Henson ES, Gibson SB. 2006. Suviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. Cellular Signaling 18(12):2089-97.

Hentschel SJ, Sawaya R. 2003. Optimizing outcomes with maximal surgical resection of malignant gliomas. Cancer Control. 10(2):109–114.

Hidalgo M, Eckhardt SG. 2001. Development of matrix metalloproteinase inhibitors in cancer therapy. J Natl Cancer Inst. 93(3):178–193.

Higuchi, M, Ohnishi T, Arita N, Hiraga S, Hayakawa T. 1993. Expression of tenascin in human gliomas: Its relation to histological malignancy. Acta Neuropathology. 85(5):481–487.

Hodgson JG, Yeh RF, Ray A, Wang NJ, Smirnov I, Yu M, Hariono S, Silber J, Feiler HS, Gray JW, Spellman PT, Vandenberg SR, Berger MS, James CD. 2009. Comparative analyses of gene copy number and mRNA expression in glioblastoma multiforme tumors and xenografts. Neuro Oncol. 11(5):477-87

Holbro T, Civenni G, Hynes NE. 2003. The ErbB receptors and their role in cancer progression. Exp Cell Res. 284(1):99-110.

Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M , Robey PG, Poole AR, Pidoux I, Ward JM, Birkedal–Hansen H. 1999. MT1–MMP–deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell. 99(1):81–92.

Hunter SB, Brat D, Olson J, Von Deimling A, Zhou W, Van Meir EG. 2003. Alterations in molecular pathways of diffusely infiltrating glial neoplasms: application to tumor classification and anti–tumor therapy (Review). Int J Oncol. 23(4):857–869.

Hynes NE, Stern DF. 1994. The biology of erbB2/neu/HER2and its role in cancer. Biochim Biophys Acta 1198(2):165–184.

Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. 2002. Human cortical glial tumors contain neural stem–like cells expressing astroglial and neuronal markers in vitro. Glia. 39(3):193–206.

Ikebe T, Shinohara M, Takeuchi H, Beppu M, Kurahara S, Nakamura S, Shirasuna K. 1999. Gelatinolytic activity of matrix metalloproteinase in tumour tissues correlates with invasiveness of oral cancer. Clin Exp Metastasis. 17(4):315–23.

Imai K, Yokohama Y, Nakanishi I, Ohuchi E, Fujii Y, Nakai N, Okada Y. 1995. Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. J. Biol. Chem. 270(12):6691–6697.

Inada M, Wang Y, Byrne MH, Rahmna MU, Miyaura C, Lopez-Otin C, Krane SM. 2004. Critical roles for collagenase-3 (MMP13) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci. USA. 101(49):17192–17197.

Ito A, Nagase H. 1988. Evidence that human rheumatoid synovial matrix metalloproteinase 3 is an endogenous activator of procollagenase. Arch. Biochem. Biophys. 267 (1):211–216.

Ito T, Ito M, Shiozawa J, Naito S, Kanematsu T, Sekine I. 1999. Expression of the MMP-1 in human pancreatic carcinoma: relationship with prognostic factor. Mod Pathol.12(7):669-674.

Itoh M, Murata T, Suzuku T, Shondoh M, Nakajima K, Imai K, Yoshida K. 2006. Requirement of STAT3 activation for maximal collagenase-1 (MMP-1) induction by epidermal growth factor and malignant characteristics in T24 bladder cancer cells. Oncogene. 25(8):1195-1204.

Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. 1998. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res. 58(5):1048–51.

Jaalinoja J, Herva R, Korpela M, Hoyhtya M, Turpeenniemi-Hujanen T. 2000. Matrix metalloproteinase 2 (MMP–2) immunoreactive protein is associated with poor grade and survival in brain neoplasms. J Neurooncol. 46(1):81–90.

Jacobs AH, Kracht LW, Gossmann A, Ruger MA, Thomas AV, Thiel A, Herholz K. 2005. Imaging in neurooncology. NeuroRx. 2(2):333–347

Jemal A, Siegal R, Ward E, Hao Y, Xu J, Thun MJ. 2009. Cancer statistics, 2009. CA Cancer J Clin. 59(4):225-249.

Jessen KR, Mirsky R. 2005. The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci 6(9):671-682.

Johnson GL, Dohlman HG, Graves LM. 2005. MAPK kinase kinases (MKKKs) as a target class for small-molecule inhibition to modulate signaling networks and gene expression. Curr Opin Chem Biol. 9(3):325-331.

Jun T, Gjoerup O, Roberts TM. 1999. Sci STKE. 1999(13):PE1

Junge CE, Lee CJ, Hubbard KB, Zhang Z, Olson JJ, Hepler JR, Brat DJ, Traynelis SF. 2004. Protease-activated receptor-1 in human brain: localization and functional expression in astrocytes. Exp Neurol. 188(1):94-103.

Kajita M, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H, Seiki M. 2001. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J Cell Biol. 153(5):893–904.

Kanaly CW, Ding D, Heimberger AB, Sampson JH. 2010. Clinical applications of a peptide–based vaccine for glioblastoma. Neurosurg Clin N Am. 21(1):95–109.

Kanamori Y, Matsushima M, Minaguchi T, Kobayashi K, Sagae S, Kudo R, Terakawa N, Nakamura Y. 1999. Correlation between expression of the matrix metalloproteinase-1 gene in ovarian cancers and an insertion/deletion polymorphism in its promoter region. Cancer Res. 59(17):4225-4227

Karin M, Liu Z, Zandi E. 1997. AP–1 function and regulation. Curr Opin Cell Biol. 9 (2):240–246.

Kargiotis O, Chetty C, Gondi CS, Tsung AJ, Dinh DH, Gujrati M, Lakka SS, Kyritsis AP, Rao JS. 2008. Adenovirus-mediated transfer of siRNA against MMP-2 mRNA results in impaired invasion and tumor-induced angiogenesis induces apoptosis in vitro and inhibits tumor growth in vivo in glioblastoma. Oncogene. 27(35):4830-40

Kato T, Kure T, Chang JH, Gabison EE, Itoh T, Itohara S, Azar DT. 2001. Diminished corneal angiogenesis in gelatinase A-deficient mice. FEBS Lett. 508(2):187–190.

Keime–Guibert F, Chinot O, Taillandier L, Cartalat-Carel S, Frenay M, Kantor G, Guillamo JS, Jadaud E, Colin P, Bondiau PY, Menei P, Loiseau H, Bernier V, Honnorat J, Barrie M, Mokhtari K, Mazeron JJ, Bissery A, Delattre JY. 2007. Radiotherapy for glioblastoma in the elderly. N Engl J Med. 356(15):1527–35.

Kieseier BC, Seifert T, Giovannoni G, Hartung HP. 1999. Matrix metalloproteinases in inflammatory demyelination: targets for treatment. Neurology. 53(1):20–5.

Kim JW, Simmer JP, Hart TC, Hart PS, Ramaswami MD, Bartlett JD, Hu JC. 2005. MMP–20 mutation in autosomal recessive pigmented hypomaturation amelogenesis imperfecta. J Med Genet. 42(3):271–275

Kim Y, Lawler S, Nowicki MO, Chiocca EA, Friedman A. 2009. A mathematical model for pattern information of glioma cells outside the tumor spheroid core. J Theor Biol. 260(3):359–371

Kita D, Yonekawa Y, Weller M, Ohgaki H. 2007. PI3KCA alterations in primary (de novo) and secondary glioblastomas. Acta Neuropathol. 113(3):295–302.

Kleihues P, Cavenee WK. 2000. WHO classification of tumors: Pathology and genetics of tumors of the nervous system. Lyon, France: *IARCpress.*

Kodate M, Kasai T, Hashimoto H, Yasumoto K, Iwata Y, Manabe H. 1997. Expression of matrix metalloproteinase (gelatinase) in T1 adenocarcinoma of the lung. Pathol Int. 47(7):461–469.

Korzus E, Nagase H, Rydell R, Travis J. 1997. The mitogen–activated protein kinase and JAK–STAT signaling pathways are required for an oncostatin M–responsive element–mediated activation of matrix metalloproteinase 1 gene expression. J Biol Chem. 272 (2) 1188–1196.

Krex D, Klink B, Hartmann C, von Deimling A, Pietsch T, Simon M, Sabel M, Steinbach JP, Heese O, Reifenberger G, Weller M, Schackert G; German Glioma Network. 2007. Long–term survival with glioblastoma multiforme. Brain. 130(Pt 10):2596–606

Kumagai K, Ohno I, Okada S, Ohkawara Y, Suzuki K, Shinya T, Nagase H, Iwata K, Shirato K. 1999. Inhibition of matrix metalloproteinases prevents allergen–induced airway inflammation in a murine model of asthma. J Immunol. 162(7):4212–4219.

Vasantha Kumar AR, Renaudin J, Wilson CB, Boldrey EB, Enot KJ, Levin V. 1974. Procarbazine hydrochloride in the treatment of brain tumors. Phase 2 study. J Neurosurg. 40(3):365–371.

Lakka SS, Gondi CS, Dinh DH, Olivero WC, Gujrati M, Rao VH, Sioka C, Rao JS. 2005. Specific interference of urokinase-type plasminogen activator receptor and matrix metalloproteinase-9 gene expression induced by double-stranded RNA results in decreased invasion, tumor growth, and angiogenesis in gliomas. J Biol Chem. 280(23):21882-21892

Lakka SS, Jasti SL, Gondi C, Boyd D, Chandrasekar N, Dinh DH, Olivero WC, Gujrati M, Rao JS. 2002. Downregulation of MMP-9 in ERK-mutated stable transfectants inhibits glioma invasion *in vitro*. Oncogene. 21(36):5601-5608

Lampert K, Machein U, Machein MR, Conca W, Peter HH, Volk B. 1998. Expression of matrix metalloproteinases and their tissue inhibitors in human brain tumors. Am J Pathol.153(2):429-437

Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela–Arispe ML. 2005. Processing of VEGF–A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol. 169(4):681–691

Li Q, Park PW, Wilson CL, Parks WC. 2002. Matrilysin shedding of syndecan–1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell. 111(5):635–646.

Libermann TA, Nusbaum HR, Razon N. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature 313(5998): 144–147

Liotta LA, Kohn EC. 2001. The microenvironment of the tumour–host interface. Nature. 411(6835):375–379.

Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature. 284(5751): 67–68.

Lipkowitz S. 2003. The role of ubiquitination –proteasome pathway in breast cancer: ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer. Breast Cancer Res. 5(1):8-15.

Liu Z, Li N, Diaz LA, Shipley M, Senior RM, Werb Z. 2005. Synergy between a plasminogen cascade and MMP–9 in autoimmune disease. J Clin Invest. 115(4):879–887.

Longva KE, Pedersen NM, HaslekasC, Stang E , Madshus IH. 2005. Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. Int. J. Cancer 116 (3):359-367.

Louis DN, Ohgaki H, Wiestler OD, Cavenee WK. 2007. WHO classification of Tumours of the Central Nervous System. Lyon, France: *International Agency for Research on Cancer*. 4th ed

Lund-Johansen M, Bjerkvig R, Humphrey PA, Bigner SH, Bigner DD, Laerum OD. 1990. Effect of epidermal growth factor on glioma cell growth, migration, and invasion in vitro. Cancer Res. 50(18):6039–6044

Mandonnet E, Delattre J, Tanguy M, Swanson K, Carpentier A, Duffau H, Cornu P, Effenterre R, EC J, Capelle LJ. 2003. Continuous growth of mean tumor diameter in a subset of grade ii gliomas. Ann Neurol. 53 (4):524–528.

Martignetti, JA, Aqeel AA, Sewairi WA, Bouman CE, Kambouris M, Mayouf SA, Sheth KV, Eid WA, Dowling O, Harris J, Glucksman MJ, Bahabri S, Meyer BF, Desnick RJ. 2001. Mutation of the matrix metalloproteinase 2 (MMP-2) gene causes a multicentric osteolysis and arthritis syndrome. Nature Genet. 28(3):261–265.

McCawley LJ, Matrisian LM. 2001. Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol. 13(5):534-540.

McCready J, Broaddus W, Sykes V, Fillmore H. 2005. Association of a single nucleotide polymorphism in the matrix metalloproteinase-1 promoter with glioblastoma. Int J Cancer 117(5):781-785.

Masson R, Lefebvre O, Noel A, Fahime ME, Chenard MP, Wendling C, Kebers F, LeMeur M, Dierich A, Foidart JM, Basset P, Rio MC. 1998. In vivo evidence that the stromelysin-3 metalloproteinase contributes in a paracrine manner to epithelial cell malignancy. J Cell Biol. 140(6):1535–1541.

McGowan KA, Bauer EA, Smith LT. 1994. Localization of type I human skin collagenase in developing embryonic and fetal skin. J InvestDermatol. 102(6):951–957.

McGuire JK, Li Q, ParksWC. 2003. Matrilysin (matrix metalloproteinase–7) mediates E–cadherin ectodomain shedding in injured lung epithelium. Am J Pathol. 162(6):1831–1843

McQuibban GA, Butler GS, Gong JH, Bendall L, Power C, Clark–Lewis I, Overall CM. 2001. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell–derived factor–1. J Biol Chem. 276(47):43503–43508.

Mehta M, Gilbert M, Mirimanoff R and Borwn P. 2004. Is combination chemoradaition the new gold standard for gliomas. *Medical Communications Media*.

Miller KD, Saphner TJ, Waterhouse DM, Chen TT, Rush-Taylor A, Sparano JA, Wolff AC, Cobleigh MA, Galbraith S, Sledge GW. 2004. A randomized phase II feasibility trial of BMS–275291 in patients with early stage breast cancer. Clin Cancer Res 10(6):1971–1975

Morris DG, Huang X, Kaminski N, Wang Y, Shapiro SD, Dolganov G, Glick A, Sheppard D. 2003. Loss of integrin α(v)β6–mediated TGF–β activation causes Mmp12–dependent emphysema. Nature 422(6928):169–173.

Murray GI, Duncan ME, Arbuckle E, Melvin WT, Fothergill JE. 1998. Matrix metalloproteinases and their inhibitors in gastric cancer. Gut. 43(6):791–797.

Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE. 1996. Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. Nat Med. 2(4):461-462.

Murray GI, Duncan ME, O'Neil P, McKay JA, MelvinWT, Fothergill JE. 1998. Matrix metalloproteinase–1 is associated with poor prognosis in oesophageal cancer. J Pathol. 185(3):256–261.

Muthuswamy SK, Gilman M, Brugge JS. 1999. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol. 19 (10):6845-6857.

Nagane M, Levitzki A, Gazit A, Cavenee WK, Huang HJ. 1998. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl–XL and caspase-3-like proteases. Proc Natl Acad Sci USA. 95(10):5724–5729

Nagarajan RP, Costello JF. 2009. Epigenetic mechanisms in glioblatoma multiforme. Semin Cancer Biol. 19(3):188–197.

Nakagawa T, Kubota T, Kabuto M, Sato K, Kawano H, Hayakawa T, Okada Y. 1994. Production of matrix metalloproteinases and tissue inhibitor of metalloproteinases-1 by human brain tumors. J of Neurosurg 81(1):69-77.

Nakano A, Tani E, Miyazaki K, Yamamoto Y, Furuyama J. 1995. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gliomas. J Neurosurg 83(2):295-307.

Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C. 1997. Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev. 23(1):35–61.

Newton HB. 1994. Primary brain tumors: review of etiology, diagnosis and treatment. Am Fam Physician. 49(4):787-797

Nikkola J, Vihinen P, Vlaykova T, Hahka–Kemppinen M, Kähäri VM, Pyrhönen S. 2002. High expression levels of collagenase-1 (MMP-1) and stromelysin-1 (MMP-3) correlate with shorter disease free survival in human metastatic melanoma. Int J Cancer 97(4):432–438.

Nikkola J, Vihinen P, Vuoristo MS, Kellokumpu-Lehtinen P, Kähäri VM, Pyrhönen S. High serum levels of matrix metalloproteinase-9 and matrix metalloproteinase-1 are associated with rapid progression in patients with metastatic melanoma. Clin Cancer Res.11(14):5158-66.

Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M. 2001. Release of an invasion promoter E–cadherin fragment by matrilysin and stromelysin-1. J Cell Sci. 114(Pt 1):111–118.

Nuttall RK, Pennington CJ, Taplin J, Wheal A, Yong VW, Forsyth PA, Edwards DR. 2003. Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells. Mol Cancer Res. 1(5):333-345.

Ogiso H, Ishitani R, Nureki O, Fukai S, Yamanaka M, Kim JH, Saito K, Sakamoto A, Inoue M, Shirouzu M, Yokoyama S. 2002. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. Cell. 110(6):775-787.

Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schuler D, Probst–Hensch NM, Maiorka PC, Baeza N, Pisani P, Yonekawa Y, Yasargil MG, Lutolf UM, Kleihues P. 2004. Genetic pathways to glioblastoma: a population-based study. Cancer Res. 64(19):6892–6899

Ohgaki H, Kleihues P. 2007. Genetic pathways to primary and secondary glioblastoma. Am J Pathol. 170(5):1445-1453.

Olayioye MA, Neve RM, Lane HA, Hynes NE. 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J. 19(13):3159-3167.

Opdenakker G, Van Damme J. 1992. Cytokines and proteases in invasive processes: molecular similarities between inflammation and cancer. Cytokine. 4(4): 251–258.

Page-McCaw A, Ewald AJ, Werb Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol. 8(3):221–233.

Papathoma AS, Petraki C, Grigorakis A, Papakonstantinou H, Karavana V, Stefanakis S, Sotsiou F, Pintzas A. 2000. Prognostic significance of matrix metalloproteinase 2 and 9 in bladder cancer. Anticancer Res. 20(3B):2009–2013.

Pardal R, Clarke MF, Morrison SJ. 2003. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer. 3(12):895–902.

Parks WC, Wilson CL, Lopez–Boado YS. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat. Rev. Immunol. 4(8):617–629.

Parney IF, Hao C, Petruk KC. 2000. Glioma immunology and immunotherapy. Neurosurgery. 46(4):778–791.

Perego C, Vanoni C, Massari S, Raimondi A, Pola S, Cattaneo M, Francolini M, Vicentini L, Pietrini G. 2002. Invasive behaviour of glioblastoma cell lines is associated with altered organisation of the cadherin-catenin adhesion system. J Cell Sci. 115 (Pt 16):3331-3340

Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC. 1997. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. J Cell Biol. 137(6):1445–1457

Provenzale JM, McGraw P, Mhatre P, Guo AC, Delong D. 2004. Peritumoral brain regions in gliomas and meningiomas: investigation with isotropic diffusion-weighted MR imaging and diffusion-tensor MR imaging. Radiology. 232(2):451–460.

Rainov NG, Soling A. 2006. Clinical studies with targeted toxins in malignant glioma. Rev Recent Clin Trials. 1(2): 119-131.

Rajah R, Nachajon RV, Collins MH, Hakonarson H, Grunstein MM, Cohen P. 1999. Elevated levels of the IGF-binding protein protease MMP-1 in asthamatic airway smooth muscle. Am J Respir Cell Mol Biol 20(20):199-208.

Rao JS. 2003. Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 3(7):489-501.

Rasheed BK, McLendon RE, Friedman HS, Friedman AH, Fuchs HE, Bigner DD, Bigner SH. 1995. Chromosome 10 deletion mapping in human gliomas: a common deletion region in 10q25. Oncogene. 10(11):2243–2246

Ravanti L, Kähäri VM. 2000. Matrix metalloproteinases in wound repair. Int. J. Mol. Med. 6(4) 391–407.

Redemann N, Holzmann B, von Ruden T, Wagner EF, Schlessinger J, Ullrich A. 1992. Anti-oncogenic activity of signaling-defective epidermal growth factor receptor mutants. Mol Cell Biol. 12(2):491-498.

Reunanen N, Li SP, Ahonen M, Foschi M, Han J, Kähäri VM. 2002. Activation of p38a MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. J Biol Chem. 277(35): 32360–32368

Riese DJ 2nd, Stern DF. 1998. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays 20(1):41-48

Robins HI, O'Neill A, Gilbert M, Olsen M, Sapiente R, Berkey B, Mehta M. 2008. Effect of dalteparin and radiation on survival and thromboembolic events in glioblastoma multiforme: a phase II ECOG trial. Cancer Chemother Pharmacol. 62(2):227–233.

Rooney PH, Murray GI, Stevenson DA, Haites NE, Cassidy J, McLeod HL. 1999. Comparative genomic hybridization and chromosomal instability in solid tumours. Br J Cancer. 80(5-6):862–873.

Rutter JL, Mitchell TI, Buttice G, Meyers J, Gusella JF, Ozelius LJ, Brinckerhoff CE. 1998. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription Cancer Res. 58(23):5321–5325.

Salah Z, Maoz M, Pizov G, Bar-Shavit R. 2007. Transcriptional regulation of human protease-activated receptor 1: a role for the early growth response-1 protein in prostate cancer. Cancer Res. 67(20):9835-9843.

Salomon DS, Brandt R,Ciardiello F, Normanno N. 1995. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol. 19(3):183-232.

Saghatelian A, Jessani N, Joseph A, Humphrey M, Cravatt BF. 2004. Activity-based probes for the proteomic profiling of metalloproteases. Proc Natl Acad Sci U S A 101(27):10000–10005.

Schlessinger J. 2002. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell 110 (6):669-672.

Schmalfeldt B, Prechtel D, Harting K, Spathe K, Rutke S, Konik E, Fridman R, Berger U, Schmitt M, Kuhn W, Lengyel E. 2001. Increased expression of matrix metalloproteinases (MMP)-2, MMP-9 and the urokinase–type plasminogen activator is associated with progression from benign to advanced ovarian cancer. Clin Cancer Res. 7(8):2396–4204

Schmidt-Ullrich RK, Mikkelsen RB, Dent P, Todd DG, Valerie K, Kavanagh BD, Contessa JN, Rorrer WK, Chen PB. 1997. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. Oncogene 15(10):1191-1197.

Schmittling RJ, Archer GE, Mitchell DA, Heimberger A, Pegram C, Herndon JE 2nd, Friedman HS, Bigner DD, Sampson JH. 2008. Detection of humoral response in patients with glioblastoma receiving EGFRvIII-KLH vaccines. J Immunol Methods. 339(1):74-81.

Sharrocks AD, Brown AL, Ling Y, Yates PR. 1997. The ETS-domain transcription factor family. Int. J. Biochem Cell Biol. 29(12):1371–1387.

Sideras K, Schaefer PL, Okuno SH, Sloan JA, Kutteh L, Fitch TR, Dakhil SR, Levitt R, Alberts SR, Morton RF, Rowland KM, Novotny PJ, Loprinzi CL. 2006. Low-molecular-weight heparin in patients with advanced cancer: a phase 3clinical trial. Mayo Clin Proc. 81(6):758–767.

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. 2003. Identification of a cancer stem cell in human brain tumors. Cancer Res 63(18):5821–5828.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. 2004. Identification of human brain tumour initiating cells. Nature. 432(7015):396-401.

Song G, Ouyang G, Bao S. 2005. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med. 9(1):59-71.

Sottrup–Jensen L, Birkedal–Hansen H. 1989. Human fibroblast collagenase-α-macroglobulin interactions. Localization of cleavage sites in the bait regions of five mammalian α– macroglobulins. J Biol Chem. 264(1):393–401

Springman EB, Angleton EL, Birkedal–Hansen H, Van Wart HE. 1990. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of Cys 73 active site zinc complex in latency and a "cystein switch" mechanism for activation. Proc Natl Acad Sci. USA. 87(1):364-368.

Stea B, Falsey R, Kislin K, Patel J, Glanzberg H, Carey S, Ambrad AA, Meuillet EJ, Martinez JD. 2003. Time and dose-dependent radiosensitisation of the glioblastoma multiforme U251 cells by the EGF receptor tyrosine kinase inhibitor ZD1839 ('Iressa'). Cancer Lett. 202(1):43–51.

Stickens D, Behonick DJ, Ortega N, Heyer B, Hartenstein B, Yu Y, Fosang AJ, Schorpp–Kistner M, Angel P, Werb Z. 2004. Altered endochondral bone development in matrix metalloproteinase 13–deficient mice. Development 131(23):5883–5895

Sternlicht M D, Kouros–Mehr H, Lu P, Werb Z. 2006. Hormonal and local control of mammary branching morphogenesis. Differentiation. 74(7):365–381.

Sternlicht MD, Werb Z. 2001. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol. 17:463-516.

Stojic J, Hagemann C, Haas S, Herbold C, Kühnel S, Gerngras S, Roggendorf W, Roosen K, Vince GH. 2008. Expression of matrix metalloproteinases MMP-1, MMP-11 and MMP-19 is correlated with the WHO-grading of human malignant gliomas. Neurosci Res. 60(1):40-49.

Stummer W, Pichlmeier U, Meinel T, Wiestler OD, Zanella F, Reulen HJ. 2006. ALA-Glioma Study Group. 2006. Fluorescence–guided surgery with 5–aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. Lancet Oncol. 7(5):392–401.

Stupp R, Gander M, Leyvraz S, Newlands E. 2001. Current and future developments in the use of temozolomide for the treatmentof brain tumours. Lancet Oncol. 2(9):552-560.

Stupp R, Mason WP, van den Bent M, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 352(10):987–996

Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR. 2005. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res. 65(16):7052-7058.

Sun Y, Sun Y, Wenger L, Rutter JL, Brinckerhoff CE, Cheung HS. 1999. p53 down-regulates human matrix metalloproteinase-1 (Collagenase-1) gene expression. J Biol Chem. 274(17):11535–11540.

Sun Y, Zeng XR, Wenger L, Firestein GS, Cheung HS. 2004. p53 down-regulates matrix metalloproteinase-1 by targeting the communications between AP-1 and the basal transcription complex. J Cell Biochem 92(2):258–269

Suzuki K, Enghild JJ, Morodomi T, Salvesen G, Nagase H. 1990. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (Stromelysin). Biochemistry. 29(44):10261–10270.

Swanson KR, Alvord EC Jr, Murray JD. 2000. A quantitative model for differential motility of gliomas in grey and white matter. Cell Prolif. 33(5):317–329.

Tamaki S, Eckert K, He D, Sutton R, Doshe M, Jain G, Tushinski R, Reitsma M, Harris B, Tsukamoto A, Gage F, Weissman I, Uchida N. 2002. Engraftment of sorted/expanded human central nervous system stem cells from fetal brain. J Neurosci Res. 69(6):976–986.

Thompson RW, Parks WC. 1996. Role of matrix metalloproteinases in abdominal aortic aneurysms. Ann N Y Acad Sci. 800:157–174

Tolcher AW, Gerson SL, Denis L, Geyer C, Hammond LA, Patnaik A, Goetz AD, Schwartz G, Edwards T, Reyderman L, Statkevich P, Cutler DL, Rowinsky EK. 2003. Marked inactivation of O6-alkylguanine DNA alkyltransferase activity with protracted temozolomide schedules. Br J Cancer. 88(7):1004–1011.

Tso CL, Freije WA, Day A, Chen Z, Merriman B, Perlina A, Lee Y, Dia EQ, Yoshimoto K, Mischel PS, Liau LM, Cloughesy TF, Nelson SF. 2006. Distinct transcription profiles of primary and secondary glioblastoma subgroups. Cancer Res. 66(1):159–167.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL. 2000. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA. 97(26):14720–14725

Vaalamo M, Karjalainen–Lindsberg ML, Puolakkainen P, Kere J, Saarialho–Kere U. 1998. Distinct expression profiles of stromelysin–2 (MMP–10), collagenase–3 (MMP–13), macrophage metalloelastase (MMP–12), and tissue inhibitor of metalloproteinase–3 (TIMP–3) in intestinal ulcerations. Am J Pathol. 152(4):1005–1014.

Van Meter TE, Broaddus WC, Rooprai HK, Pilkington GJ, Fillmore HL. 2004. Induction of membrane-type-1 matrix metalloproteinase by epidermal growth factor-mediated signaling in gliomas. Neuro-Oncol 6(3):188-199.

VanMeter TE, Rooprai HK, Kibble MM, Fillmore HL, Broaddus WC, Pilkington GJ. 2001. The role of matrix metalloproteinase genes in glioma invasion: co-dependent and interactive proteolysis. J Neurooncol. 53(2):213-235.

Vihinen P, Kähäri VM. 2002. Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer. 99(2):157-166.

Vince GH, Wagner S, Pietsch T, Klein R, Goldbrunner RH, Roosen K, Tonn JC. 1999. Heterogeneous regional expression patterns of matrix metalloproteinases in human malignant gliomas. Int J Dev Neurosci. 17(5-6):437-445.

Vincenti MP. 2001. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Matrix Metalloproteinase protocols.

Vincenti MP, Coon CI, Lee O, Brinckerhoff CE. 1994. Regulation of collagenase gene expression by IL-1b requires transcriptional and post–transcriptional mechanisms. Nucleic Acids Res. 22(22):4818-4827.

Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. 1998. MMP–9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell. 93(3):411–422.

Walker MD, Strike TA, Sheline GE. 1979. An analysis of dose–effect relationship in the radiotherapy of malignant gliomas. Int J Radiat Oncol Biol Phys. 5(10):1725–31.

Wang M, Liu YE, Greene J, Sheng S, Fuchs A, Rosen EM, Shi YE. 1997. Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. Oncogene 14(23):2767–2774.

Wang J, Sakariassen PO, Tsinkalovsky O, Immervoll H, Boe SO, Svendsen A, Prestegarden L, Rosland G, Thorsen F, Stuhr L, Molven A, Bjerkvig R, Enger PO. 2008. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. Int J Cancer. 122(4):761–768.

Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, Ohgaki H. 1996. Over-expression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol. 6(3):217–224.

Westermarck J, Seth A, Kähäri VM. 1997. Differential regulation of interstitial collagenase (MMP-1) gene expression by ETS transcription factors. Oncogene 14(22): 2651–2660

White LA, Mitchell TI, Brinckerhoff CE. 2000. Transforming growth factor beta inhibitory element in the rabbit matrix metalloproteinase-1 (collagenase-1) gene functions as a repressor of constitutive transcription. Biochim Biophys Acta 1490(3): 259–268.

Whitelock JM, Murdoch AD, Lozzo RV, Underwood PA. 1996. The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenases plasmin and heparanases. J Biol Chem. 271(17): 10079–10086.

Wilhelm SM, Eisen AZ, Teter M, Clarck SD, Kronberger A, Goldberg G. 1986. Human fibroblast collagenase: glycosylation and tissue-specific levels of enzyme synthesis. Proc. Natl. Acad. Sci. USA 83(11): 3756–3760

Wilson CB, Gutin P, Boldrey EB, Crafts D, Levin VA, Enot KJ. 1976. Single–agent chemotherapy of brain tumors. A five–year review. Arch Neurol. 33(11): 739-744.

Wilson CL, Heppner KJ, Labosky PA, Hogan BL, Matrisian LM. 1997. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci USA. 94(4):1402–1407.

Wilson CL, Ouellette AJ, Satchell DP, Ayabe T, Lopez–Boado YS, Stratman JL, Hultgren SJ, Matrisian LM, Parks WC. 1999. Regulation of intestinal–defensin activation by the metalloproteinase matrilysin in innate host defense. Science. 286(5437): 113–117.

Woessner JF, Nagase H. 2000. *Matrix Metalloproteinases and TIMPs*. New York: Oxford Univ. Press

Wong AJ, Ruppert JM, Bigner SH. 1992. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc Natl Acad Sci U SA 89(7): 2965-2969

Woodgett JR. 2005. Recent Advances in protein kinase B signaling pathway. Curr. Opin Cell Biol.17 (2):150-157

Wrensch M, Minn Y, Chew T, Bondy M, Berger MS. 2002. Epidemiology of primary brain tumors: current concepts and review of the literature. Neuro Oncol. 4(4):278–299

Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW. 1993. Inhibition of EGF-activated MAP Kinase signaling pathway by adenosine 3', 5'-monophosphate. Science 262(5136): 1065-1069.

Wu MP, Tamada JA, Brem H, Langer R. 1994. In vivo versus in vitro degradation of controlled release polymers for intracranial surgical therapy. J Biomed Mater Res 28(3): 387-395.

Wyatt CA, Geoghegan JC, Brinckerhoff CE. 2005. Short hairpin RNA-mediated inhibition of matrix metalloproteinase-1 in MDA-231 cells: effects on matrix destruction and tumor growth. Cancer Res. 65(23):11101-11108.

Xia H, Qi Y, Ng SS, Chen X, Li D, Chen S, Ge R, Jiang S, Li G, Chen Y, He ML, Kung HF, Lai L, Lin MC. 2009. microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs. Brain Res. 1269:158-165.

Yamamoto M, Mahanam S, Sawaya R, Fuller GN, Seiki M, Sato H, Gokaslan ZL, Liotta LA, Nicolson GL and Rao JS. 1996. Differential expression of membrane-type

matrix metalloproteinase and its correlation with gelatinase A activation in human malignant brain tumors *in vivo* and *in vitro*. Cancer Res. 56(2):384–392

Yamoutpour F, Bodempudi V, Park SE, Pan W, Mauzy MJ, Kratzke RA, Dudek A, Potter DA, Woo RA, O'Rourke DM, Tindall DJ, Farassati F. 2008. Gene silencing for epidermal growth factor receptor variant III induces cell-specific cytotoxicity. Mol Cancer Ther. 7(11):3586-3597.

Ye S, Dhillon S, Turner SJ, Bateman AC, Theaker JM, Pickering RM, Day I, Howell WM. 2001. Invasiveness of cutaneous malignant melanoma is influenced by matrix metalloproteinase 1 gene polymorphism. Cancer Res. 61(4): 1296–1298

Yong VW, Power C, Forsyth P, Edwards DR. 2001. Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci. 2(7): 502–511.

Yu JS, Liu G, Ying H, Yong WH, Black KL, Wheeler CJ. 2004. Vaccination with tumor lysatepulsed dendritic cells elicits antigen–specific, cytotoxic T-cells in patients with malignant glioma. Cancer Res. 64(14): 4973–4979.

Yu Q, Stamenkovic I. 2000. Cell surface–localized matrix metalloproteinase–9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes Dev. 14(2):163-176.

Yung WK, Albright RE, Olson J, Fredericks R, Fink K, Prados MD, Brada M, Spence A, Hohl RJ, Shapiro W, Glantz M, Greenberg H, Selker RG, Vick NA, Rampling R, Friedman H, Phillips P, Bruner J, Yue N, Osoba D, Zaknoen S, Levin VA. 2000. A phase II study of temozolomide vs. procarbazine in patients with glioblastoma multiforme at first relapse. Br J Cancer. 83(5): 588-593.

Zagzag D, Friedlander DR, Miller DC, Dosik J, Cangiarella J, Kostianovsky M, Cohen H, Grumet M, Greco MA. 1995. Tenascin expression in astrocytomas correlates with angiogenesis. Cancer Res. 55(4):907–914.

Zagzag D, Friedlander DR, Dosik J, Chikramane S, Chan W, Greco MA, Allen JC, Dorovini-Zis K, Grumet M. 1996. Tenascin-C expression by angiogenic vessels in human astrocytomas and by human brain endothelial cells in vitro. Cancer Res. 56(1): 182–189.

Zandi R, Larsen AB, Andersen P, Stockhausen MT, Poulsen HS. 2007. Mechanisms for oncogenic activation of epidermal growth factor receptor. Cell Signal. 19(10): 2013-2023.

Zhang J, Sarkar S, Yong VW. 2005. The chemokine stromal cell derived factor-1 (CXCL12) promotes glioma invasiveness through MT2-matrix metalloproteinase. Carcinogenesis. 26(12): 2069-2077.

Zhang W, Wang H, Song SW, Fuller GN. 2002. Insulin-like growth factor binding protein 2: gene expression microarrays and the hypothesis-generation paradigm. Brain Pathol. 12(1): 87-94.

Zhu Y, Parada LF. 2002. The molecular and genetic basis of neurological tumours. Nat Rev Cancer. 2(8):616-626

Zimmermann S, Moelling K. 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 286(5445):1741-1744.

Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K. 2000. Impaired endochondral ossification and angiogenesis in

mice deficient in membrane-type matrix metalloproteinase I. Proc. Natl Acad. Sci. USA. 97(8):4052–4057

APPENDICES

Appendix A:

Cloning strategy for MMP-1

MMP-1 was purchased in the pOTB7 vector that had been modified with attB sites. This would allow cloning into an entry vector (pDONR221) using the Gateway system by Invitrogen. Once the entry clone is obtained, MMP-1 can be moved into a variety of destination vectors using the Invitrogen Gateway cloning system. The pIRES2Ac-GFP, a vector from Clontech, has been converted to a destination vector and was used for this project.

MMP-1/pOTB7 and pDONR221 DNA (1:1 molar ratio) was set up in a BP reaction and 1ul was transformed into electrocompetent α–select cells from Bioline. The BP reaction is mediated by BP clonase enzymes composed of Integrase and Integrase host factor (IHF) proteins that facilitate the recombination between DNA clones containing att**B** sites and a donor vector with att**P** sites. The cells were plated on kanamycin plates (50ug/ml LB agar). Colonies were analyzed by the Epicenter Colony Fast-Screen Kit (Cat#FS0472H). The correct colony was struck on another kanamycin LB (Luria Broth) agar plate and a single colony was used for inoculation of a miniprep culture. DNA was isolated using the Fermentas GeneJet Miniprep Kit (Cat#K0503). The DNA was digested with NsiI enzyme that would disrupt the kanamycin gene of the pDONR221 backbone. The DNA was purified using the PCR cleanup procedure of the Promega Wizard SV Gel and PCR Cleanup System (Cat#A9282)

MMP-1/pDONR221 DNA and pIRES2Ac-GFP/rfa DNA (1:1 molar ratio) was set up in an LR reaction and 1µl was transformed into electrocompetent α–select cells from Bioline. In an LR reaction, the integrase, IHF and excisionase (Xis) enzymes catalyze the recombination between entry clone (carrying gene of interest flanked with att**L** sites) and a destination vector with att**R** sites. The cells were plated on kanamycin plates (50ug/ml LB agar). Colonies were analyzed by the Epicenter Colony Fast-Screen Kit (Cat#FS0472H). The correct colony was struck on another kanamycin LB agar plate and a single colony was used for inoculation of a day culture. An overnight maxiprep culture was inoculated from the day culture. DNA was isolated using the Invitrogen PureLink HiPure Maxiprep Kit (Cat# K210007).

BP Reaction Creating a Gateway® entry clone from an *att*B-flanked PCR product Add the following components to a 1.5 ml tube at room temperature and mix: attB-PCR product (=10 ng/µl; final amount ~15-150 ng) 1-7 µl Donor vector (150 ng/µl) 1 µl TE buffer, pH 8.0 to 8 µl

1. Thaw on ice the BP Clonase™ II enzyme mix for about 2 minutes. Vortex the BP Clonase™ II enzyme mix briefly twice (2 seconds each time).
2. To each sample (Step 1, above), add 2 µl of BP Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
3. Return BP Clonase™ II enzyme mix to -20°C or -80°C storage.
4. Incubate reactions at 25°C for 1 hour.

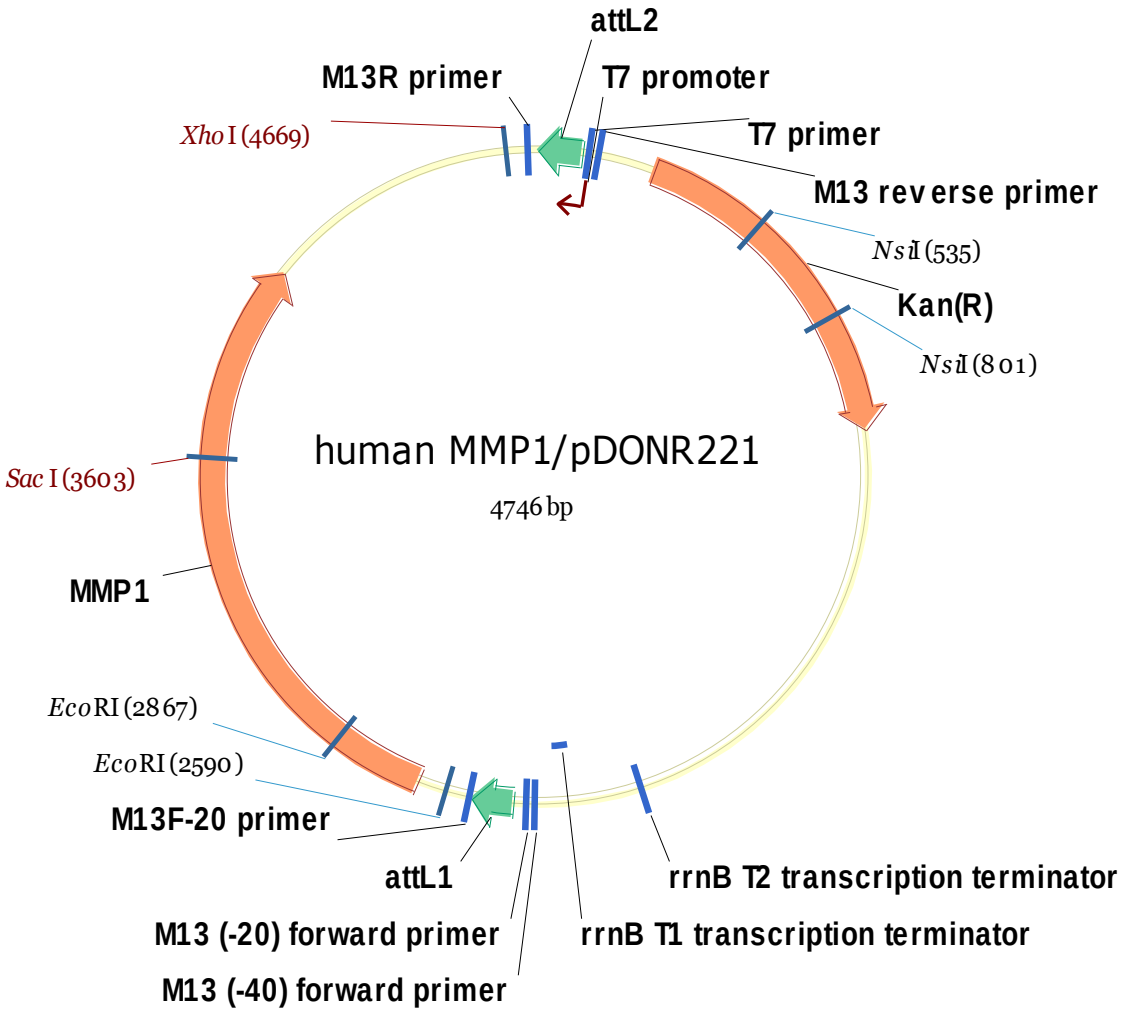
5. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

LR Reaction Transferring the gene from a Gateway® entry clone to destination vector

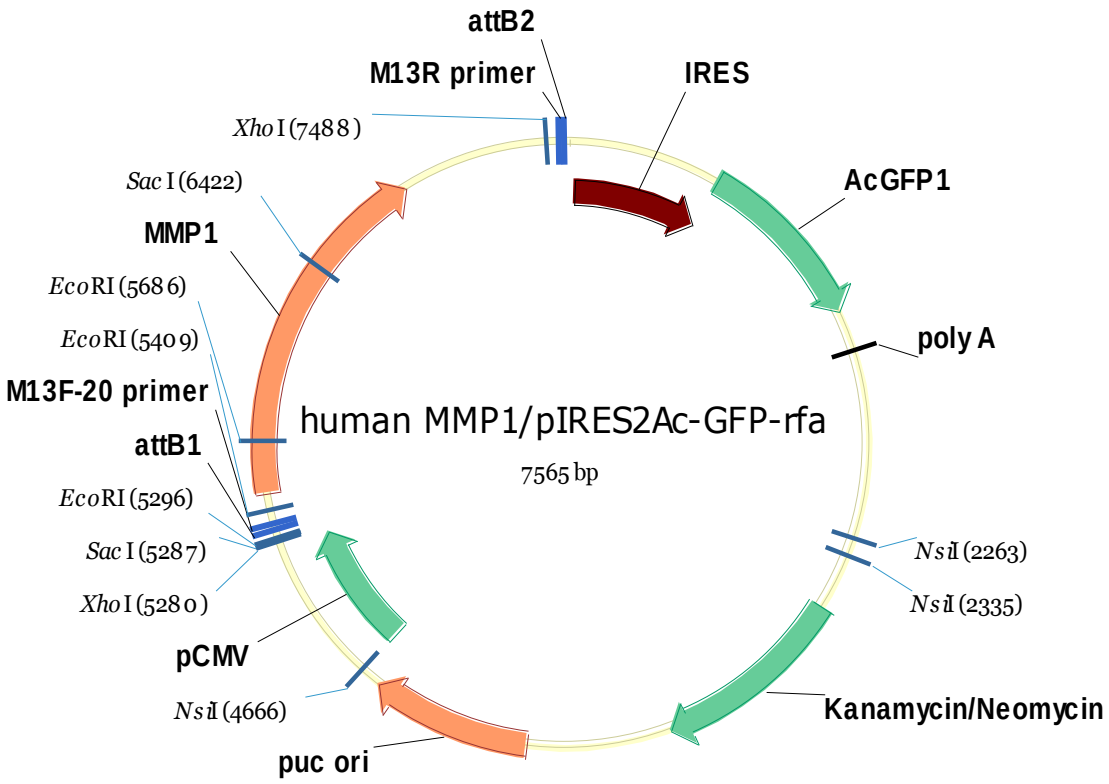
1. Add the following components to a 1.5 ml tube at room temperature and mix: Entry clone (50-150 ng) 1-7 µl
2. Destination vector (150 ng/µl) 1 µl, TE buffer, pH 8.0 to 8 µl
3. Thaw on ice the LR Clonase ™ II enzyme mix for about 2 minutes. Vortex the LR Clonase ™ II enzyme mix briefly twice (2 seconds each time).
4. To each sample (Step 1, above), add 2 µl of LR Clonase ™II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
5. Return LR Clonase ™ II enzyme mix to -20°C or -80°C storage.
6. Incubate reactions at 25°C for 1 hour.
7. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Cloning and molecular biology procedures were performed at the VCU – Massey Cancer Center Molecular Biology Core Facility, supported, in part, with funding from NIH-NCI CCSG Center core grant (2-P30-CA-16059)

Appendix B: Vector Map of Donor Vector



Appendix C: Vector Map of Destination Vector



Appendix D

Protocol for activation of MMP-1 by APMA

All solutions were freshly made on the day of performing the assay. MMP-1 present in the conditioned media samples of U251MG and U87MG GBM cells that were stably over-expressed with pIRES-MMP-1-AcGFP were treated with amino-phenyl mercuric acid (APMA).

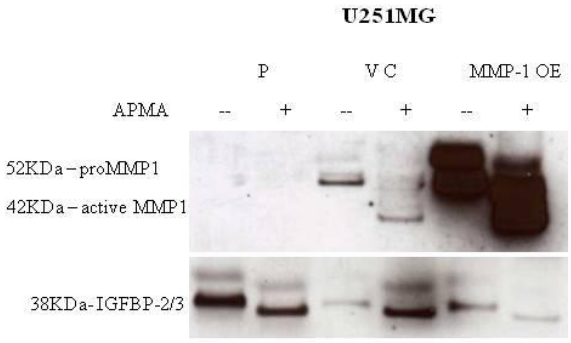
A stock solution of 10 mM APMA was prepared by dissolving 35.2 mg of APMA in 0.1M sodium hydroxide (NaOH). Tris-Triton-calcium (TTC) buffer was prepared with final concentration of 50mM of Tris-HCL, 1mM of calcium chloride and 0.05% of Triton-X-100. 20 µl of conditioned media containing pro-MMP-1 from each cell line was added to 200 µl of APMA (final concentration 4mM) and 280 µl of TTC buffer. The reaction mix was incubated at 37°C water-bath for 24 hours.

For immunoblots, the conditioned media was used as it is at the end of incubation for MMP-1 analysis. For treatment in cell cultures, the APMA was removed by dialysis using Amicon Ultracentrigufal filter devices. Before addition of the reaction mix, the filters were blocked with 100 µl of 1mg/ml of bovine serum albumin (BSA) for 30 min at 37°C. Thereafter, 500 µl of sterile PBS was added and the filters were centrifuged at 11,000 x g for 20 minutes at 4°C. The filters were inverted and spun briefly to remove residual PBS and then dried. The reaction mixture was added to these filters and centrifuged at 11,000 x g for 20 minutes at 4°C to remove APMA. This was repeated so as to remove the maximal amount of APMA. The concentration of proteins in

conditioned media was then analyzed using Biorad-DC protein assay. Equal amount of protein was used for treatment of cells in culture.

Appendix E

IGFBP2/3 does not get cleaved in the presence of activated MMP-1 from U251MG MMP-1 overexpressing cells.

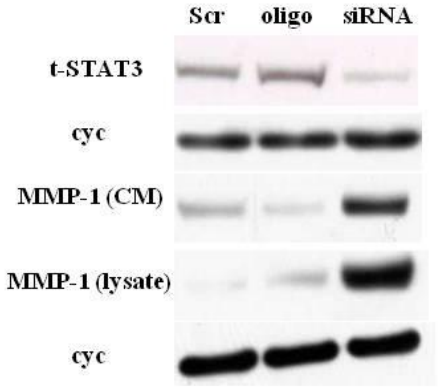


Active MMP-1 in U251MG cells and IGFBP-2/3 levels

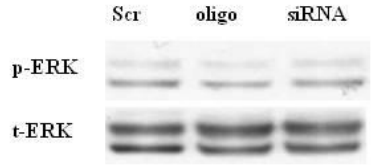
In the above figure, conditioned media from U251MG cells (Parentals P, Vector control VC, MMP-1 over-expressers MMP-1 OE) was extracted and concentrated. MMP-1 was activated using APMA and levels of IGFBP2/3 were examined. The data shows that MMP-1 gets activated with APMA treatment. There is no affect on IGFBP-2/3 by activated MMP-1. APMA treatment, by itself, resulted in molecular weight shift as seen in P and VC samples.

Appendix F

STAT-3 siRNA treatment in T98G cells leads to MMP-1 induciton



Inhibition of STAT3 (T98G) and MMP-1 levels



ERK levels in STAT3 siRNA treated cells (T98G)

T98G cells treated with STAT3 siRNA show a significant knockdown of STAT3 (total) protein levels. In these samples, MMP-1 expression was observed to be remarkably high as compared to scrambled (scr) and oligofectamine (oligo) (transfection reagent) controls. Lower panel figure shows that the levels of phospho- and total ERK do not change among the three sample groups- Scrambled, Ogofectamine and STAT3 siRNA-treated T98G cells.

VITA

Monika Anand was born in New Delhi, India and is a citizen of India. She received her Bachelor in Science (B.Sc) degree from Maitreyi College, University of Delhi, Delhi, India in 1998 and then completed her Master in Science (M.Sc) (Biotechnology) from University of Calicut, Calicut, India in 2000. She worked as trainee at Tata Energy Research Institute (TERI), New Delhi, India for six months. Thereafter, she joined Polyamine and Transgenics Lab, Department of Genetics, University of Delhi, India as Research Assistant. She worked in Department of Plant Molecular Biology at University of Delhi for a year before coming to USA for pursuing doctorate degree. Monika joined Ph.D. program in Department of Biochemistry and Molecular Biology at Virginia Commonwealth University in the fall semester of 2004.