Virginia Commonwealth University
VCU Scholars Compass

Theses and Dissertations

Graduate School

2006

The Influence of a Single Nucleotide Polymorphism In The Matrix Metalloproteinase-1 Promoter on Glioma Biology

Jessica McCready
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

Part of the Nervous System Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/1123

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.

www.manaraa.com

© Jessica McCready 2006

All Rights Reserved

THE EFFECT OF A SINGLE NUCLEOTIDE POLYMORPHISM IN THE MATRIX METALLOPROTEINASE-1 PROMOTER ON GLIOMA BIOLOGY

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

By

JESSICA McCREADY
B.S., Cornell University, 1998

Director: HELEN L. FILLMORE
Assistant Professor Department of Neurosurgery
Adjunct Assistant Professor Department of Anatomy and Neurobiology

Virginia Commonwealth University
Richmond, Virginia
May 2006

ACKNOWLEDGEMENTS

I would like to thank the many people who have helped me achieve my goal. Firstly, my advisor, Helen Fillmore, for the time, effort and support she has given me over the past four years. Also, Zendra Zehner, who met with me many times to discuss data and far exceeded the role of committee member. I would like to thank the other members of my committee including William Broaddus, who supported me throughout this process, Joyce Lloyd who met with me many times to discuss experimental design and controls, and Linda Phillips who taught me a great deal in manuscript and grant writing. I would like to thank the members of the Broaddus and Fillmore Labs, especially Virginia Sykes for her technical support and training and Aaron Clark for our discussions in the lab. I would like to thank the Department of Anatomy and Neurobiology not only for their financial support but also for creating rigorous academic requirements that challenged me from the beginning of the degree program. I would like to acknowledge the Department of Neurosurgery and the Hord, Cullather and Crone families for their financial support. Lastly, I would like to thank my family, especially my husband, Justin Levine.

TABLE OF CONTENTS

Page

Acknowledgements ii

List of Tables v

List of Figures vi

List of Abbreviations vii

Abstract ix

Chapter

1 Introduction 1

1.1 Brain Tumors 2

1.1.1 Overview of Brain Tumors 2

1.1.2 Therapy for Gliomas 5

1.1.3 Glioma Biology 10

1.2 Matrix Metalloproteinases 11

1.2.1 Members 11

1.2.2 Structure 12

1.2.3 Physiological and Pathological Roles 14

1.2.4 Regulation of MMPs 21

1.3 Matrix Metalloproteinase-1 23

1.3.1 MMP-1 and the Central Nervous System 23

1.3.2 Single Nucleotide Polymorphism 26

1.3.3 MMP-1 Regulation 34

1.3.4 ETS Transcription Factors 37

1.3.5 AP-1 Transcription Factors 43

1.4 Hepatocyte Growth Factor/Scatter Factor 45

1.4.1 Overview 45

1.4.2 Signal Transduction 53

1.4.3 Hepatocyte Growth Factor/Scatter Factor and Gliomas 59

2 Materials and Methods 62

2.1 Primary Human Samples 63

2.2 Genotyping 64

2.3 Sequencing 64

2.4 Nuclear Extraction 65

2.5 Electrophoretic Mobility Shift Assay 66

2.6 Transient Transfection and Luciferase Assay 66

2.7 RNA Extraction and Real Time PCR 67

2.8 Protein Extraction 69

2.9 ELISA 70

2.10 Immunoblotting 71

2.11 Chromatin Immunoprecipitation ... 71

3 Association of a Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 Promoter with Glioblastoma (IJC 2005 117:781-785) ... 76
3.1 Abstract ... 77
3.2 Introduction ... 78
3.3 Materials and Methods ... 80
3.3.1 Cell Lines ... 80
3.3.2 Primary Human Samples ... 80
3.3.3 Genotyping ... 80
3.3.4 Sequencing ... 81
3.3.5 Nuclear Extraction and Electrophoretic Shift Assays ... 81
3.3.6 Transfection and Luciferase Assays ... 82
3.3.7 RNA Extraction and Real Time PCR ... 82
3.3.8 Protein Extraction and ELISA ... 82
3.3.9 Statistical Analyses ... 83
3.4 Results ... 84
3.5 Discussion ... 96

4 Increase in MMP-1 2G Promoter Transcription from Hepatocyte Growth Factor Stimulation is caused by AP1 and Ets-1 cooperativity in Gliomas ... 99
4.1 Abstract ... 100
4.2 Introduction ... 101
4.3 Results ... 105
4.4 Discussion ... 121
4.5 Materials and Methods ... 128
4.5.1 Cell Lines and Culture Conditions ... 128
4.5.2 Genotyping and Sequencing ... 128
4.5.3 Immunoblotting ... 128
4.5.4 Chromatin Immunoprecipitation ... 128
4.5.5 Transfection and Luciferase Assays ... 129
4.5.6 RNA Extraction and Real Time PCR ... 129

5 Discussion ... 131

References ... 142

Appendices ... 155
I. DNA-Protein Interactions ... 156
i. DNA-Protein Affinity Pull Down Assay Protocol ... 156
ii. DNA-Protein Affinity Pull Down Assay cFos ... 158
iii. Electromobility Assay 1G probe ... 159
iv. Electromobility Shift Assay Supershift ... 160
II. Promoter Activitation ... 161
i. 1G promoter -/+ HGF/SF ... 161
ii. 2G promoter -/+ HGF/SF ... 162

Vita ... 163

List of Tables

Page

Table 1. Members of the MMP family 13

Table 2. The ETS-domain family 38

Table 3. Members of the AP-1 family 44

Table 4. Relationship between MMP-1 promoter polymorphism and glioblastoma 87

Table 5. Comparison of allele frequency between DNA from high grade glioma samples generated in this study with DNA from healthy volunteers from previously published studies 88

List of Figures

Page

Figure 1. Schematic of the structure of the MMP family members 15

Figure 2. Schematic of the single nucleotide polymorphism in the MMP-1 promoter 29

Figure 3. Schematic of the structure of the members of Ets family proteins 39

Figure 4. Schematic of hepatocyte growth factor/scatter factor 47

Figure 5. Schematic of the met tyrosine kinase receptor 49

Figure 6. Schematic of the met signaling pathway 55

Figure 7. Organization of mammalian MAPK cascade 57

Figure 8. Genotyping of the MMP-1 promoter polymorphism in healthy individuals and individuals with glioblastoma 85

Figure 9. Binding interactions of the polymorphism in the MMP-1 promoter 89

Figure 10. Transcriptional activation of the MMP-1 promoter 92

Figure 11. MMP-1 mRNA and protein levels in normal brain and GBM samples of differing genotype 94

Figure 12. Glioma cell lines differ in their MMP-1 promoter status 106

Figure 13. MMP-1 genotype determines responsiveness to HGF/SF treatment in glioma call lines 108

Figure 14. The ERK kinase is activated in U251 glioma cells after HGF treatment 111

Figure 15. Response of MMP-1 2G promoter to HGF is more marked when compared to the MMP-1 1G promoter response 119

Figure 16. AP-1 proteins are synthesized in response to HGF treatment in U251 cells 116

Figure 17. Binding pattern of ETS and AP-1 proteins to the 2G MMP-1 distal promoter is different than that of the 1G MMP-1 distal promoter 118

Figure 18. Schematic of the proteins bound to the MMP-1 distal promoter as determined by chromatin immunoprecipitation 124

List of Abbreviations

AP-1 activator protein 1

APMA amino phenyl mercuric acid

ATF activating transcription factor

BMK big MAP kinase

bZIP basic leucine zipper

CDK cyclin dependent kinase

CNS central nervous system

EAE experimental autoimmune encephalomyelitis

ECM extracellular matrix

EGFR epidermal growth factor receptor

EMSA electrophoretic mobility shift assay

EMT epithelial mesenchymal transition

ERK extracellular signal related kinase

ETS E26 transformation specific or E twenty six

GBM glioblastoma multiforme

HGF/SF hepatocyte growth factor/scatter factor

JNK cJun N-terminal kinase

LOH loss of heterozygosity

MAF musculoaponeurotic fibrosarcoma

MAPK mitogen activated protein kinase

MMP matrix metalloproteinase

MS multiple sclerosis

PCV procarbazine, carmustine and vincristine

PDGF platelet derived growth factor

PMA phorbol myristic acetate

PSP progressive supranuclear palsy

PTEN phosphatase and tensin homology

RB retinoblastoma

RRE Ras responsive element

SNP single nucleotide polymorphism

TIMP tissue inhibitor of metalloproteinase

TMZ temozolomide

ABSTRACT

THE INFLUENCE OF A SINGLE NUCLEOTIDE POLYMORPHISM IN THE MATRIX METALLOPROTEINASE-1 PROMOTER ON GLIOMA BIOLOGY

Jessica McCready

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

Virginia Commonwealth University, 2006

Director: Helen L. Fillmore, Ph.D. Assistant Professor Department of Neurosurgery, Adjunct Assistant Professor Department of Anatomy and Neurobiology

Glioblastomas are an incurable type of brain tumor with a mean survival time of 9-12 months following diagnosis. One of the reasons for this poor prognosis is the ability of tumor cells to invade the surrounding normal brain tissue. Enzymes responsible for this invasive nature include the matrix metalloproteinase family. MMP-1 is a member of this family which has been well studied in many types of invasive tumors, with gliomas being an exception. We studied a single nucleotide polymorphism (SNP) in the MMP-1 promoter that may influence glioma biology. This SNP consists of the presence (2G) or absence (1G) of a guanine nucleotide at position -1607. The additional guanine nucleotide creates a binding site for ETS transcription factors and combined with the AP-1 binding site at position -1602 creates a Ras Responsive Element. We determined that the distribution of the MMP-1 genotype differed significantly between the healthy population and the glioblastoma patient population, with the 2G/2G genotype more prevalent in the glioblastoma patients. In addition, MMP-1 mRNA and protein examined in a select group of patient tissue had significantly higher levels when compared to normal brain controls, however, there was no correlation with genotype. Promoter reporter assays indicated that the 2G promoter

was approximately three times more active than the 1G promoter in three different glioma cell lines.

We investigated potential signaling mechanisms responsible for increases in MMP-1 transcription due to the presence of the RAS responsive element. Treatment of glioma cell lines with hepatocyte growth factor/scatter factor (HGF/SF) led to significant increases in MMP-1 transcription, via the MAP kinase ERK pathway. AP-1 transcription factor proteins, cJun and cFos were increased in response to HGF treatment but not Ets-1 and ETV-1. HGF/SF treatment of glioma cell lines differing in their MMP-1 genotype affected binding of ETS and AP-1 proteins to the endogenous MMP-1 distal promoter. Using chromatin immunoprecipitation assays, we identified these differentially DNA-bound AP-1 and ETS proteins.

The data presented indicate that the MMP-1 SNP (-1607) is important in glioma biology and may contribute to tumor function and future investigations into its role in glioma biology is warranted.

Chapter 1

Introduction

1.1 Brain Tumors

1.1.1 Overview of Brain Tumors

This section will present a general overview of adult cancers, with a focus on gliomas, which account for approximately 86% of all malignant brain tumors. These include the astrocytoma, oligodendroglioma and glioblastoma. Malignant brain tumors make up 2% of all cancers in adults. This number is much higher for children as brain tumors are the second most common cancer and the most common solid tumor in childhood[1]. The incidences of brain tumors in both children under the age of 14 and in the elderly over the age of 70[2] are increasing and unfortunately these tumors, in most cases, remain incurable.

In the general population of the United States, gliomas occur at a rate of 5-10 per 100,000[2]. The incidence of gliomas increases, as in other cancers, in individuals older than 30 years of age. In general, males are more likely than females to have a brain tumor, with a male to female ratio of 1.5:1. Most brain tumors occur in the supratentorial region of the brain, in the frontal, temporal or parietal lobes, specifically[1]. Currently, little is known about the risk factors for gliomas. Many studies have been conducted, investigating such items as cell phones or head trauma for possible roles in the etiology of brain tumors, but none has been found to consistently associate with their incidence. Gliomas are thought to arise as a result of an accumulation of genetic alterations resulting in the ability of the cells to escape checkpoints designed to prevent uncontrolled cell growth. Therefore any agent that damages DNA, such as a chemical, physical or biological agent, can be considered to be a neurocarcinogen. One environmental agent that has been associated with increases in brain tumors in adults is ionizing radiation used as a treatment for childhood tumors and leukemia[1].

In 1926, Bailey and Cushing began a classification system that is still used, in a modified form, today. They named the tumors after the type of cell that the tumor most resembled histologically, either in the developing embryo or in the adult[1]. In certain tumor types the cells may be so atypical in appearance, with similarities to multiple mature cell types, that they were difficult to name; an example is the glioblastoma, named for a theoretical glial precursor cell that is not actually seen in embryogenesis. In an attempt to address and reduce some of the confusion, the World Health Organization has added and modified the systems started by Bailey and Cushing as well as other prominent neuropathologists. They identified four malignancy grades and named them I-IV. The biologically benign pilocytic astrocytoma was classified as a Grade I astrocytoma. Diffuse astrocytomas, a low grade malignancy, were designated as Grade II. The high grade gliomas, anaplastic astrocytoma and glioblastoma multiforme (GBM), were designated Grades III and IV respectively[2-4]. There are four main features that neuropathologists use to determine tumor grade: 1) nuclear atypia 2) mitoses 3) microvascular proliferation and 4) necrosis[2,4]. In certain tumors, immunocytochemistry can aid in the classification if the tumor cells express an antigen that a particular cell type typically expresses[3].

There are four main symptoms of brain tumors. Partial or generalized seizures usually indicate cortical impingement by a neoplasm that is often slow growing. Raised intracranial pressure in the unyielding skull can lead to displacement of either cerebrospinal fluid or intracranial blood volume. This presents itself clinically as headaches, nausea, vomiting, drowsiness, or visual abnormalities. Focal neurological deficits are another symptom of brain tumors. They can be motor or sensory in nature if the tumor is supratentorial. Cranial nerve palsies or cerebellar dysfunction tend to

occur if the tumor is located in the posterior fossa. The fourth main symptom of brain tumors is cognitive dysfunction , a symptom usually due to supratentorial tumors or posterior fossa tumors that cause osbstructive hydrocephalus[2].

If the physician suspects that a brain tumor is the cause of the patient's symptoms, an MRI with and without the contrast agent, gadolinium, is currently the best method to define the characteristics of the tumor. MR spectroscopy can be useful to distinguish a tumor from a non-neoplastic mass[2]. The diagnosis must be confirmed by a histological examination of a biopsy sample and is based on the most malignant portion of the tumor[2,3].

The survival rate for patients with brain tumors has been gradually increasing over the past thirty years, with the exception of the GBM. This tumor has the poorest survival rate of all brain tumors with a median survival of 9-12 months irrespective of treatment modalities[1,2,4,5]. There are several factors that affect patient survival. The Karnofsky Performance Scale (0-100) rates the patient's overall functioning level. A higher score can be related to a better prognosis. In adults, a younger patient has a better chance of survival than an older patient. Females have a higher rate of survival than males. The location of the tumor and the extent to which the neurosurgeon can resect the tumor also affect patient survival[1]. Gliomas rarely spread beyond the central nervous system (CNS), thus the primary determinant of clinical outcome is tumor grade. The biologically benign grade I tumor, if in a location allowing complete surgical resection, is curable. Grade II astrocytomas are low grade malignancies that have relatively long survival rates but are not curable by surgery alone. A patient with a grade III astrocytoma will likely succumb to the tumor within a few years and the grade

IV astrocytoma, which are commonly resistant to any type of therapy leads to patient death within 9-12 months following diagnosis[4].

1.1.2 Therapy for Gliomas

After reading the above information, it is clear that the diagnosis of a high grade glioma is tantamount to a death sentence. Currently there are no treatment options that are curative, with the median survival rate between nine and twelve months and only 3.3% of patients surviving to the 5 year point[5]. The standard treatment for patients with these tumors consists of surgery followed by radiation and, in some cases, chemotherapy. This treatment regimen only confers modest benefit to the patient, but without any treatment the median survival time is reduced to three months. This section will describe, in detail, each of these treatment modalities and the benefit realized by the patient.

Although surgery is the usual first line of treatment for high grade glioma patients, it, by itself, is not curative due to the invasive nature of these tumors. By the time the diagnosis has been made, the tumor cells have usually invaded into the normal CNS and cannot all be removed by surgery[6]. Following the imaging of the tumor with an MRI, the neurosurgeon will analyze the patient to see if he or she is a good candidate for surgery. A common way to judge this is to analyze the response of the patient to corticosteroid treatment, such as dexamethasone, which reduces inflammation and edema in the brain. If the patient is in good condition while receiving 16 mg per day of dexamethasone, extensive surgical debulking may be of some benefit, but if the patient is in poor condition and the dexamethasone does little to alleviate the symptoms, surgery is unlikely to achieve any further benefit[7]. There are

benefits to the patient if he or she is a candidate for surgery. Surgical debulking of the tumor can immediately alleviate the symptoms caused by the tumor mass, namely the intracranial pressure likely to cause headache, vomiting and decreased cognition[6,7]. Surgery may also help alleviate the onset of seizures that are not responsive to drug therapy and it is thought to facilitate the effectiveness of the other therapies because the hypoxic and necrotic core tends to be highly resistant to radiation and chemotherapy[6]. One of the causes of death from these tumors is from the raised intracranial pressure, therefore extensive resection of the tumor can prolong the patient's life. However, if the patient lives long enough, almost all high grade gliomas will spread throughout the brain and begin to involve vital structures and this cannot be changed or cured by surgery.

There have been several reports that evaluated the effect of surgery on survival, and aside from the increase in the quality of life, no long-term survival benefits have been found. It is known that a wider resection margin taken during surgery will delay the time to recurrence and that more extensive resection is associated with a longer survival but it is still only a modest benefit of approximately six months survival. If the patient is over 65 years at the time of surgery that survival benefit drops to only three months[7]. These reports must be viewed with caution because surgeons may tend to select healthier patients or those with more accessible tumors for more aggressive surgery, i.e. wider resection margin. The factors associated with a healthier patient include high Karnofsky rating, young age, minor, if any, neurological deficits, and location in a non-eloquent area.

There have been major advancements in technology that have aided the neurosurgeon over the last twenty years. Conventional surgery debulks the tumor and

is stopped when normal brain is reached either visually or by tissue consistency. This can be difficult and unreliable, especially in the GBM which has no clear tumor margin. Image guided neurosurgery using preoperative imaging data is now commonly used to identify the extent of the tumor. Functional MRI data can also be used to define the eloquent areas of the brain, such as motor, speech, visual and sensory tracts. Certain operating rooms use imaging techniques including ultrasound, CT and MRI, during the operation to help increase the area of resection while leaving normal brain intact. A newer technology currently in a Phase III clinical trial boasts a one to eight month survival benefit. 5-aminolevulinic acid is taken preoperatively by the patient. This compound is taken up by the glioma cells and converted to the fluorescent protoporphyrin IX which is visible through a standard illuminating operating microscope[7]. This greatly enhances the surgeon's ability to see and therefore remove the tumor mass and to be able to distinguish normal brain from tumor.

Surgery is usually followed by radiotherapy in most patients with a high grade glioma. A typical dose of conventional radiation consists of a total of 60 Gy delivered at 2 Gy per fraction to the tumor mass plus a two to three centimeter margin surrounding the tumor[6]. The Brain Tumor Study Group conducted a study in 1978 and the results from that study make it clear that radiation therapy is the best adjuvant therapy for GBM patients; however, the maximum tolerated dose is well below the dose required for long term tumor control[6,8]. As a therapy for GBMs, radiation only confers a modest improvement in median survival, from three months, with no treatment, to nine to twelve months.

There have been attempts to improve the efficacy of radiation treatment by both technical and biological means. Brachytherapy is one example of the technical

improvements to conventional radiotherapy. Brachytherapy is radiotherapy delivered by placing a radioactive source directly into the tumor[8]. It has shown little added benefit in terms of either tumor recurrence or patient survival although it may confer modest increases in quality of life for patients who have an inoperable deep seated tumor[8]. One pitfall of brachytherapy is that only 10-30% of tumor patients are eligible to undergo this treatment and 50-80% of patients that received brachytherapy had a local recurrence of a malignant glioma within 2 cm of the original tumor mass. This statistic illustrates that a therapy that offers local treatment for a disease that infiltrates the brain should be used in combination with other therapies targeting infiltrative tumor cells[8].

There have been over thirty years of clinical trials designed to test the efficacy of systemic chemotherapy on high grade gliomas. All of them have had disappointing results in both their impact on recurrence and patient survival. These studies tested the most commonly used agents, either nitrosourea based compounds such as BCNU (carmustine) or the combination therapy of procarbazine, carmustine and vincristine, commonly called PCV. A modest increase of 6% in the one year survival was reported regardless of the chemotherapeutic agent used[6]. A pitfall with the PCV therapy is the severe side effects experienced by the patients. They include prolonged myelosuppression, skin reactions and hepatotoxicity, sometimes so troublesome that the patient must stop the therapy[9]. A newer chemotherapeutic agent, temozolomide (TMZ) is at least as effective as the nitrosoureas but appears to have less of the side effects associated with PCV. It is an oral cytotoxic alkylating agent that is able to cross the blood brain barrier and prevent the replication of rapidly dividing cells[6]. It adds a methyl group to a guanine base that must be removed by methyl guanine

methyltransferase. Once the methyl guanine methyltransferase enzyme is depleted, the methyl group can not be excised, cell cycle checkpoints are activated and cells with intact p53 will undergo apoptosis. A phase III clinical trial conducted by the European Organization for Research in the Treatment of Cancer and the National Cancer Institute of Canada compared adjuvant chemotherapy with TMZ plus radiation therapy to radiation alone in patients with newly diagnosed GBMs[6]. The median survival was 12 months for patients receiving radiation alone compared with 15 months for the patients receiving the combination therapy. There was also an improvement in two year survival from 10% to 26%. The results from this study indicate that TMZ is well tolerated and is at least as effective as the often used nitrosoureas but with fewer of the harmful side effects[9].

Other therapeutic options that have been investigated include the design of drug delivery systems intended to circumvent problems with reagents crossing the blood brain barrier, thereby permitting delivery of high concentrations of drug without the systemic toxicities seen with the standard therapies. These include polymer wafer implants and catheters directly implanted into the brain through the skull[9]. The wafers contain BCNU and have been shown to increase survival by two months without any significant changes in toxicity to the patient. Phase I clinical trials are currently being conducted to test the effect of CpG oligonucleotides on recurrent GBMs[10] via convection enhanced delivery. The side effects were not serious and included worsening of neurological deficits and fever. There have been other studies evaluating convection enhanced delivery conducted as clinical trials that indicate convection enhanced delivery is a viable treatment option in the future. Examples include the evaluation of transferrin conjugated to a genetically modified diphtheria toxin[11],

interleukin-13 conjugated with a truncated Pseudomonas exotoxin[12], paclitaxel[13] and a chimeric protein composed of TGF alpha and mutated Pseudomonas exotoxin[14].

Scientists have been researching this disease for decades with very little, if any, improvements in patient survival. With a better understanding of the biological basis of gliomas, therapies based on the molecular profile of the tumor might allow at least a modest success in the treatment of these tumors.

1.1.3 Glioma Biology

Glioblastoma can arise in one of two ways[2-4,15]. A primary, or *de novo*, GBM usually presents itself in older patients without any evidence of prior clinical disease. It is a highly aggressive, invasive tumor. A secondary GBM is usually seen in younger patients who have been treated or are presumed to have had (on the basis of history and imaging studies) a low grade astrocytoma (grade II) that transforms into a GBM. 70% of low grade gliomas will progress to a GBM within 5-10 years of the original diagnosis regardless of prior treatments[5]. The primary GBM is much more common than the secondary GBM thus the secondary GBM is not as well studied. A patient with a secondary GBM will frequently have been treated with either radiotherapy, cytotoxic drugs or a combination of the two[3]. Primary and secondary GBMs are two distinct clinical entities that develop along different genetic pathways but have very similar clinical, genetic and biological similarities. Once the low grade glioma has progressed to the high grade glioma and is then called the secondary GBM, it is clinically indistinguishable from the primary GBM. There is no distinction between the two types in regards to neuropathologic identification, proliferation, invasion and resistance to all of the available treatment methods[4]. It is now known that the same

genetic pathways appear to be targeted in both primary and secondary GBMs, but the components of the pathway such as the proteins or genes involved may not be identical. The relevant pathways have been reviewed previously and therefore will not be discussed herein. Examples of the most well characterized pathways that are altered in GBMs include PDGF, p53, RB, EGFR and PTEN[2-4,15].

1.2 Matrix Metalloproteinases

1.2.1 Members

The Matrix Metalloproteinase (MMP) family of proteins consists of 25 members (Table 1) that degrade components in the extracellular matrix (ECM). MMP-1, the founding member was discovered 46 years ago by Jerome Gross and colleagues who described the degradation of the tadpole tail during morphogenesis[16]. Since that discovery, MMPs have gotten much attention not only in cancer research but also in embryonic development, tissue morphogenesis, wound repair, and inflammatory diseases. MMPs received their name because of their dependence on metal ions for catalytic activity and their ability to degrade components in the ECM. They also have similar evolutionary paths that distinguish them from other metalloproteinases. Each member of the MMP family is generated by a distinct gene[17]. They are proteolytic enzymes that cleave internal peptide bonds and as such are classified as endopeptidases. MMPs can be referred to by either the numeric nomenclature (i.e. MMP-1) or by the common name (i.e. Collagenase-1). Historically the MMPs were divided into four groups on the basis of substrate specificity. They were grouped into the collagenases, gelatinases, stromelysins and matrilysins. The collagenases degrade structural collagens such as collagen types I, II, and III while the gelatinases

degrade type IV collagen and have a limited ability to degrade stromal collagens. Stromelysins degrade non collagen matrix molecules such as proteoglycans, laminin, fibronectin, and can also activate latent MMP family members[18]. The list of proteins belonging to this family grew so large that the classification system had to be modified. The new system groups them according to protein structure. The nomenclature changed at this time as well, and was switched to naming them sequentially. Eight structural classes were formed: five for the secreted proteins and three for the membrane type[19]. Table 1 lists the structural class for each MMP. A more in depth discussion of the structure of these proteins follows in the next section.

1.2.2 Structure

There are eight structural classes of MMPs (Figure 1). Five of these classes are secreted proteins and three are membrane type. All members of the MMP family contain a conserved zinc binding motif HEXXHXXGXXH and have a fairly conserved overall structure. There are four main domains that compose the proteins of the MMP family. All MMPs contain an N terminal pre domain, also known as the signal domain, which is responsible for directing the synthesis of the protein at the endoplasmic reticulum. This domain is removed after the synthesis. The propeptide prodomain is responsible for maintaining enzyme latency until it is either removed or disrupted. The conserved cysteine within the pro domain bridges the zinc within the catalytic domain thus preventing enzymatic activity. The linkage can be chemically disrupted by amino phenyl mercuric acid (APMA) or through proteolytic cleavage. The removal or disruption of the cysteine is referred to as the cysteine switch[20]. Certain MMPs contain

TABLE 1. Members of the MMP family.

MMP	Common Name	Structural Class
MMP-1	Collagenase-1	Simple hemopexin domain
MMP-2	Gelatinase A	Gelatin-binding
MMP-3	Stromelysin-1	Simple hemopexin domain
MMP-7	Matrilysin	Minimal domain
MMP-8	Collagenase-2	Simple hemopexin domain
MMP-9	Gelatinase B	Gelatin-binding
MMP-10	Stromelysin-2	Simple hemopexin domain
MMP-11	Stromelysin-3	Furin-activated and secreted
MMP-12	Macrophage metalloelastase	Simple hemopexin domain
MMP-13	Collagenase-3	Simple hemopexin domain
MMP-14	MT1-MMP	Transmembrane
MMP-15	MT2-MMP	Transmembrane
MMP-16	MT3-MMP	Transmembrane
MMP-17	MT4-MMP	GPI-linked
MMP-18	Collagenase-4 (*Xenopus*)	Simple hemopexin domain
MMP-19	RASI-1	Simple hemopexin domain
MMP-20	Enamelysin	Simple hemopexin domain
MMP-21	XMMP (*Xenopus*)	Vitronectin-like insert
MMP-22	CMMP (chicken)	Simple hemopexin domain
MMP-23		Type II transmembrane
MMP-24	MT5-MMP	Transmembrane
MMP-25	MT6-MMP	GPI-linked
MMP-26	Matrilysin-2	Minimal domain
MMP-27		Simple hemopexin domain
MMP28	Epilysin	Furin-activated and secreted

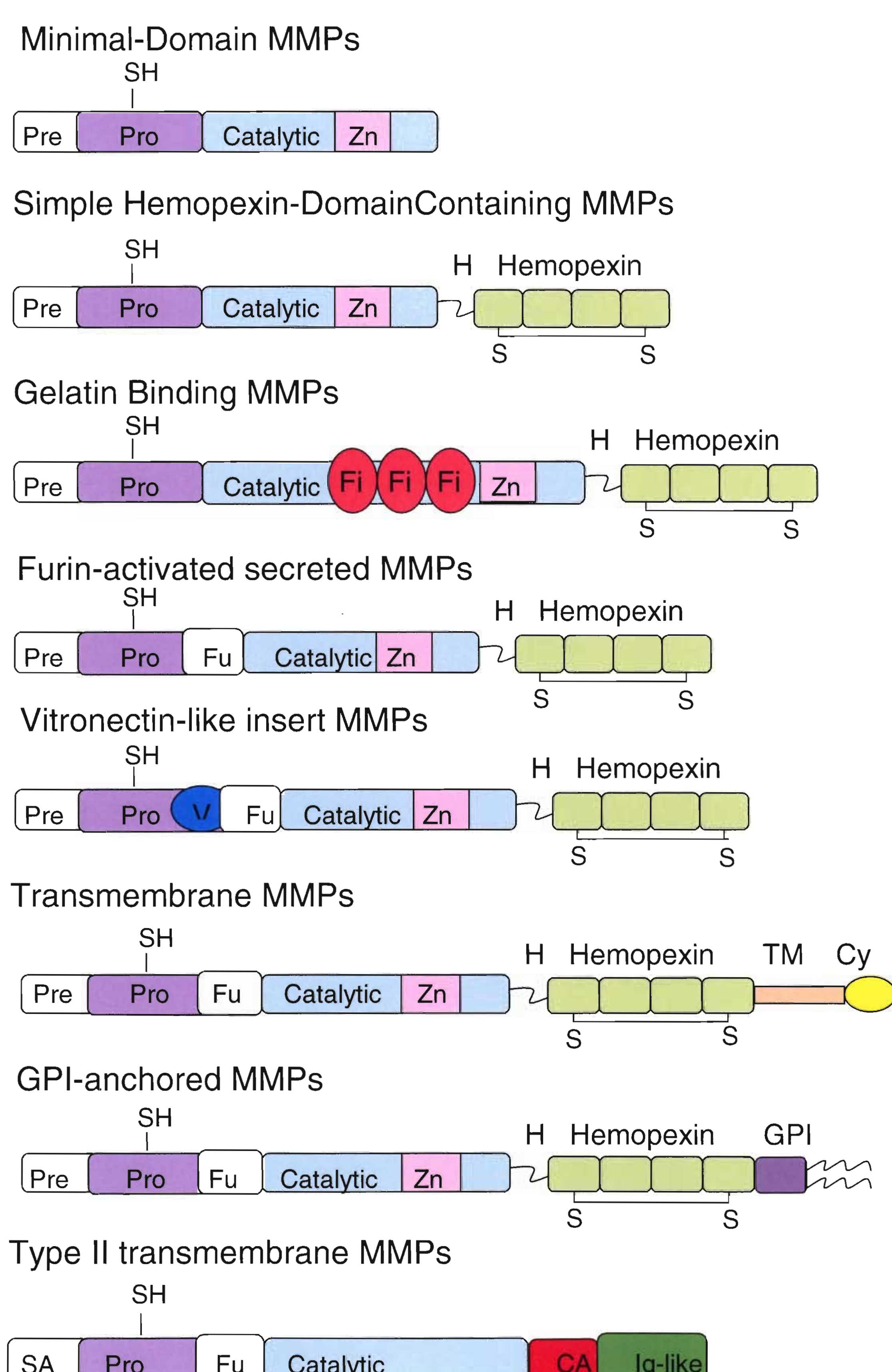
a basic insert in the propeptide domain that is cleaved by furin-like proprotein convertase proteases[17].

The catalytic domain contains the conserved zinc binding region. This domain dictates cleavage site specificity through its active site cleft and through secondary substrate binding sites located outside the active site itself. MMP2 and MMP9 have an insertion of three head to tail cysteine rich repeats within the catalytic domain that are required to bind to and cleave collagen and elastin. The hemopexin domain is connected to the catalytic domain by a hinge or linker region. This domain influences tissue inhibitor of metalloproteinase (TIMP) binding and the binding of certain substrates, membrane activation and some proteolytic activities. The hinge region varies in length and composition among the MMPs and it can influence substrate specificity. MMP9 has a unique type V collagen like insert of unknown importance at the end of the hinge region. Certain membrane type MMPs have a single pass transmembrane domain and a short cytoplasmic C terminal tail and others are linked to the membrane via a GPI anchoring domain[21].

1.2.3 Physiological and Pathological Roles

As stated above, MMP-1 was first discovered as the enzyme necessary to break down collagen in the tail of a tadpole during tissue morphogenesis some forty years ago. Since this discovery, not only have other MMPs been discovered but the many roles they play in both normal physiological states and in diseased pathological states have become clearer. Currently it is known that MMPs are important in cell migration, invasion, proliferation, apoptosis and developmental processes. These processes are needed in both physiologic and pathologic states.

Figure 1. Schematic of the structure of the MMP family members. MMPs are divided into eight structural groups, five of which are secreted and three of which are membrane type. Pre. amino terminal signal sequence; Pro, propeptide; SH, thiol group; Zn, zinc binding site; H, hinge; S-S, disulfide bond; Fi, fibronectin; Fu, intracellular furin like serine proteases; Vn, vitronectin like insert; TM, transmembrane domain; Cy, cytoplasmic domain; GPI, glycosylphosphatidylinositol anchored; SA, amino terminal signal anchor; CA, cysteine array; Ig, immunoglobulin. This figure was adapted from Egeblad and Werb Nature Reviews Cancer 2002 2:161-174


Minimal-Domain MMPs
SH
Pre
Pro
Catalytic
Zn
Simple Hemopexin-DomainContaining MMPs
SH
H
Hemopexin
Pre
Pro
Catalytic
Zn
S
S
Gelatin Binding MMPs
SH
H
Hemopexin
Pre
Pro
Catalytic
Fi
Fi
Fi
Zn
S
S
Furin-activated secreted MMPs
SH
H
Hemopexin
Pre
Pro
Fu
Catalytic
Zn
S
S
Vitronectin-like insert MMPs
SH
H
Hemopexin
Pre
Pro
V
Fu
Catalytic
Zn
S
S
Transmembrane MMPs
SH
H
Hemopexin
TM
Cy
Pre
Pro
Fu
Catalytic
Zn
S
S
GPI-anchored MMPs
SH
H
Hemopexin
GPI
Pre
Pro
Fu
Catalytic
Zn
S
S
Type II transmembrane MMPs
SH
SA
Pro
Fu
Catalytic
CA
Ig-like

There are three main activities in which MMPs are necessary in physiologic states. These include the degradation of ECM molecules to allow cell migration, alteration of the ECM microenvironment resulting in changes in cellular behavior, and modulating the activity of biologically active molecules. This last function can occur in one of two ways, either by directly cleaving molecules resulting in their activation or by modulating the activity of their inhibitors and releasing bound substrates from their stores[22]. Since MMPs degrade a variety of substrates in the ECM it is not surprising that they function as "clearing" enzymes during cellular migration. They modulate the function of many biologically active molecules that function in chemoattraction during migration. For example, the migration of epithelial, mesenchymal and neuronal cells can be affected by MMPs. During embryo implantation, trophoblasts must invade the maternal decidua. The invading cells express high levels of MMP-9 and inhibitors to this MMP prevent invasion into the decidua[23].

MMPs are necessary for cellular migration in a variety of organ systems. For example, MMPs are required for endothelial cell migration into the surrounding ECM during angiogenesis[24]. The migration of osteoclasts, the cells that remove existing bone and cartilage, during bone remodeling is dependent upon MMPs. Inhibition of MMPs prevents movement of osteoclasts into cartilage during early long bone development[25]. In order for keratinocytes to migrate during epithelial morphogenesis, cleavage of type I collagen is required, a function of MMP-1. Migration on laminin 5 by breast epithelial cells correlates with expression of both MT1-MMP and MMP-2[26].

MMPs also function in the alteration of cellular behavior. There is evidence that MMPs affect cellular proliferation, survival, apoptosis, differentiation and cellular

organization. For example, adipocytes and pancreatic epithelial cells form into three-dimensional structures. If MMP-2 is inhibited, the cells will differentiate, but in the case of the pancreatic cells, islets do not form, thereby modifying the behavior of the cell[27].

One of the main functions of MMPs is the cleavage of molecules in the ECM. This cleavage can have many results, one of which, discussed above, creates space for cells to move through the cleared ECM. Cleavage of a molecule in the ECM can generate biologically active fragments. For example, the cleavage of plasminogen by MMP-3,-7,-9-and MMP-12 results in an active molecule called angiostatin. Angiostatin is an angiogenic inhibitor. Other angiogenic inhibitors are generated as fragments from the cleavage of ECM molecules. Endostatin is a fragment of the cleavage of collagen type XVIII and the cleavage of antithrombin III results in a molecule that is an inhibitor of angiogenesis. MMPs can also regulate the bioavailability of molecules by releasing proteins bound to ECM proteins. Specific examples of this include the cleavage of the proteoglycan perlecan into smaller inactive fragments by MMP-1 and MMP-3 to release active FGF[28], cleavage of decorin by MMP-2,-3,-7 to release TGF-β, and cleavage of IGF binding proteins to release active IGF. MMP-9 cleaves IGF-BP3 which releases IGF-1 and results in the regulation of cellular proliferation via an autocrine response[29]. Cellular proliferation is also controlled by cleavage of IGF-BP1 by MMP-3[30]. This cleavage releases IGF1 which then binds to the IGF-1 receptor resulting in increased cellular proliferation. Cleavage of molecules by MMPs in the ECM can also regulate activity of the molecule by proteolytically activating or inactivating the molecule. For example, cleavage of the IL-1β precursor by MMP-2,-3,-9 results in an active form of the molecule and cleavage of endothelin-1 by MMP-2 results in greater activity as a vasoconstrictor than before the cleavage[22].

Another function of MMPs in the normal physiologic state is aiding in wound healing through keratinocyte migration and dermal contraction. MMP-1 has been implicated in keratinocyte migration and MMP-3 has been implicated in dermal contraction. MMP-3 null mice do not form a contractile ring of actin that is necessary to contract the wound. This leads to a delayed healing response in those mice[22].

MMPs are also involved in development of the bone and mammary tissue. MMP-9 is necessary for vascular invasion into the hypertrophic cartilage zone during endochondral bone formation[21]. MT1-MMP deficient mice have craniofacial dysmorphisms due to impaired intramembraneous bone formation. MMPs are an important part of mammary development because a significant amount of ECM remodeling must take place for virgin development, lactation and weaning[22].

The many beneficial roles of MMPs just described are due to their proteolysis function. The cleavage of proteins can also have detrimental affects as MMPs are known to be involved in certain diseases. The dermatological disease, bullous pemphigoid, is an autoimmune disease which causes blisters to form on the entire body. MMP-9 is upregulated in individuals with this disease and mice that do not have MMP-9 are resistant to blistering. It is now known that the initiation of blistering is caused by the recognition of collagen type XVII (BP180) by antbodies. Neutrophil elastase is the enzyme responsible for cleaving BP180. Its endogenous inhibitor, α1-PI, can be inactivated by MMP-9. Since MMP-9 inactivates α1-PI the neutrophil elastase is no longer inhibited and this permits the neutrophil elastase to cleave the BP180 and initiate blistering on the skin[21].

MMPs are also implicated in pulmonary emphysema. 90% of the inflammatory cells in patients with this disease are macrophages which secrete MMP-2,-7,-9,-12.

These MMPs can degrade elastin and inactivate α1-PI. In this instance, smokers lacking α1-PI are predisposed to get emphysema. One of the main causes of emphysema is the irreversible enlargement of peripheral air spaces. This occurs as a result of the degradation of interstitial elastic fibers by MMPs[21].

MMPs are thought to play a role in the development of intracranial and aortic aneurysms through the cleavage of elastin or fibrillin in the walls of the blood vessels leading to a breakdown in the vessel wall. MMP-2,-9,-12 are highly expressed within aortic aneurysms; MMP-12 specifically localizes to residual elastic fiber fragments. A bone marrow transplant from MMP-9 deficient donors to wild type hosts can confer a resistance to aneurysm in mice. This resistance can be reversed if the donor has wild type MMP-9 and the host is deficient for the enzyme[31].

The disease that MMPs are probably most associated with is cancer, notably cancer progression, invasion and metastasis. MMPs are usually present in greater amounts in malignant cancers, their highest expression being in the area involved in active invasion. This is not surprising as cancer cells must cross multiple ECM barriers to cross the epithelial basement membrane to enter the blood or lymph vessels and metastasize to a distant site[21]. One example of MMP involvement in invasion is the cleavage of CD44 by MT1-MMP. This releases the cell not only from neighboring cells but also from the matrix it was entwined with. MT1-MMP also helps localize MMP-9 and MMP-2 to the cell surface which is required for tumor invasion and angiogenesis[19].

MMPs are involved in other areas of cancer development and progression apart from its role in cell motility. They are responsible, in part, for the regulation of apoptosis in tumors. It is known that MMP-3 is responsible for the induction of apoptosis in mammary epithelial cells however the mechanism is still under study[32].

MMP-7 releases FASL, a protein that stimulates the death receptor FAS, from the cell surface in splenocytes[33]. Once released FAS induces apoptosis in neighboring cells. MMP-7 can also inhibit apoptosis by cleaving pro heparin binding EGF to generate mature heparin binding EGF[19,34]. This stimulates the ERBB4 receptor tyrosine kinase thereby promoting cell survival. As mentioned above MMPs can also regulate angiogenesis through the cleavage of certain proteins to release fragments that are angiogenic inhibitors.

1.2.4 Regulation

MMPs are synthesized as zymogens, secreted inactive precursor enzymes that must be cleaved for activation. The zymogen is kept inactive by the interaction of a cysteine sulfhydryl group in the propeptide domain with a zinc ion in the catalytic domain. Disruption of this bond either through chemical or proteolytic cleavage results in the removal of the propeptide domain (cysteine switch) and ultimately enzyme activation. Most MMPs are activated outside the cell either by other activated MMPs or by serine proteases. MMPs are regulated at three main levels: the transcriptional and post transcriptional levels, the protein level via enzyme activation and by inhibitors and cell surface localization. Most MMPs are tightly regulated at the transcriptional level, the exception being MMP2. It is constitutively expressed and is controlled through enzyme activation. The expression of the MMP genes is regulated by many stimulatory or suppressive factors that can affect signal transduction pathways[21]. It can also be regulated by UV radiation, inflammatory cytokines and growth factors[20,21]. A common way cytokines and growth factors regulate MMP transcription is through the induction of c-Jun and c-Fos which then bind to AP1 sites within many MMP

promoters. There are many *cis* regulatory elements that regulate MMPs with AP1 being the most important. It is present in many MMP genes allowing the induction of MMP promoters by phorbol esters. The AP1 element has been shown to acts synergistically with adjacent ETS binding sites in many MMP promoters[21]. Gene expression may also be regulated by single nucleotide polymorphisms (SNP) present in many MMP promoters such as MMP-1,-2,-3,-9,-12, The effect of a SNP in the MMP-1 promoter will be discussed in a subsequent section. MMPs can be regulated via post transcriptional methods as well, for example, MMP-1 and MMP-3 mRNA is stablilized by both phorbol esters and EGF[21].

Most MMPs are constitutively secreted once they become translated as zymogens at the endoplasmic reticulum. The next level of regulation is the level of zymogen activation. The latency of the enzyme is maintained by the interaction between the cysteine sulfhydryl group in the propetide domain and the zinc in the catalytic domain. The sulfhydryl group is the fourth ligand for the zinc ion in the active site. Enzyme activation is achieved by disruption of the cysteine-zinc interaction caused by removal of the propeptide domain. The displaced thiol group is then replaced by a water molecule that can attack a peptide bond of the MMP substrates[21].

There are three endogenous inhibitors that tightly control MMP activity, α2 macroglobulin, TIMPs, and reversion inducing cysteine rich protein with kazal motifs (RECK)[16,19,21]. The main inhibitor of MMPs in tissue fluids is α2 macroglobulin and it is produced by hepatocytes and macrophages. It is an irreversible inhibitor that binds the MMP to form a complex that then binds to a scavenger receptor, located on the low density lipoprotein receptor related protein[35]. The internalization of this MMP-inhibitor-receptor complex results in MMP degradation and is then cleared from the cell by

endocytosis. The TIMPs are reversible inhibitors with a molecular weight between 20 and 29 kDa that bind to the activated catalytic site of MMPs in a 1:1 stoichiometric fashion. The activity of TIMPs varies because they are not expressed equally in all tissues and because certain TIMPs can only inhibit certain MMPs[16]. The third inhibitor of MMPs is RECK. This protein is the only known membrane bound MMP inhibitor[19].

1.3 Matrix Metalloproteinase-1

1.3.1 MMP-1 and the Central Nervous System

MMP-1 is the founding member of the MMP family[16]. It has other names such as collagenase-1, fibroblast collagenase and interstitial collagenase. This final name is the official EC name and its EC number is EC3.4.24.7. It belongs to the simple hemopexin domain structural group which was described above. Like all other non-membrane type MMPs it is synthesized and secreted as a proenzyme or zymogen. In its unglycosylated form, which is the major form of the enzyme, it is 57 kDa. The MMP-1 gene contains 10 exons and is located chromosome at 11q22.2-22.3[36]. The regulation of the protein will be discussed in a subsequent section.

MMP-1 is involved in certain disease states in the CNS. For many of the diseases, the contribution of MMP-1 to either the development or progression of the disease is currently unclear. This section will focus on the expression of MMP-1 in the CNS.

Multiple sclerosis (MS) is characterized by repetitive disruptions of the blood brain barrier thereby allowing infiltration of mononuclear cells as well as leakage of plasma proteins into the CNS. It is thought that MS is initiated through the activation of autoreactive T cells in the periphery which migrate to the CNS because the early MS

lesion has a large number of activated T cell lymphocytes and monocytes/macrophages. These cells have been implicated as potential mediators in the breakdown of the blood brain barrier and in the formation of the inflammatory demyelinating lesions[37,38]. One of the key functions for MMPs in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE, an animal model for studying MS) could be to facilitate leukocyte entry into the CNS. An examination of the expression levels of MMP-1 in the blood of patients with MS reveals that MMP-1 mRNA is increased in the monocyte population but not in the B cells or the T cells[37]. Evaluation of the movement of these cells indicates that a significantly higher number of monocytes are able to cross the blood brain barrier when compared to T cells[38]. This data suggests that MMP-1 is associated with the movement of monocytes across the blood brain barrier into the CNS. Once across this barrier the monocyte becomes a macrophage and is a prominent contributor to the neuroinflammation seen in MS[38].

MMP-1 has been shown to be elevated in classical inflammatory states in the CNS such as MS. There is also evidence to suggest that there is an ongoing inflammatory process involved in the biology of Alzheimer's disease. This led Leake *et al.*[39] to investigate the possible connection between Alzheimer's and MMP-1 expression. The authors determined through ELISA on postmortem brain that the levels of MMP-1 were increased 50% in the diseased group when compared to the control group. MMP-1 was found in the parietal, frontal and occipital lobes of the brain but not in the temporal lobe. The authors looked to one of the pathologies of the disease to explain this increase in MMP-1. In Alzheimer's disease there are abnormal deposits of collagen along the blood vessel wall leading to thickened vessels in the cerebrum. The proposed mechanism for the increase in MMP-1 in Alzheimer's

disease is the increase in collagen since contact with type I collagen will stimulate MMP-1 transcription[40] and therefore causes an increase in MMP-1 mRNA.

Another disease of the CNS that contains elevated MMP-1 levels is progressive supranuclear palsy (PSP). It is characterized by the appearance of supranuclear gaze palsy in addition to postural instability, truncal dystonia, parkinsonism and dementia. There are neurofibrillary filaments and tau positive tangles leading to extensive neuronal degeneration and gliosis in multiple nuclei in the basal ganglia, forebrain and the brainstem[41]. Evaluation of tissue samples from patients with PSP indicates that MMP-1 protein levels are increased in the area of the substantia nigra but not in the frontal cortex. The authors offered this data as an observation and currently there is no explanation as to the stimulus or possible function related to the increased MMP-1 protein levels.

The addition of MMP-1 to organotypic spinal cord cultures, which contain a relatively intact/similar ECM as the CNS, increases cellular death in those cultures. If MMP-1 is added to dissociated cultures of human neurons, neuronal death is also increased when compared to controls. Interestingly, the addition of MMP-1 does not significantly affect cell death in cultured human fetal astrocytes[42]. The authors suggest two possible explanations for MMP-1 mediated cell death: 1) MMP-1 could be targeting one or more of proteins within the ECM that are critical to neuronal survival and their release from the ECM could result in cellular death or 2) MMP-1 could be toxic through its ability to break down type I collagen[42]. In a subsequent publication, the authors investigated the mechanism responsible for MMP-1 induced neuronal cell death. The data suggests that MMP-1 is affecting integrin signaling in a manner independent of MMP-1's proteolytic activity[43]. MMP-1 is bound to the α2β1 integrin

receptor which leads to dephosphorylation of Akt, a kinase that, when inactive, has been implicated in apoptosis. The authors propose that the complex composed of the α2β1 integrin receptor and MMP-1 begins a signaling pathway that dephosphorylates Akt thereby initiating a caspase mediated apoptosis cascade resulting in neuronal cell death.

Lastly, MMP-1 has been shown to be overexpressed in brain tumors[44-46]. This overexpression can be correlated to tumor grade. Analysis by immunohistochemistry indicates that neither neuronal cells nor glial cells express MMP-1 in normal brain, grade I or grade II astrocytomas. Analysis of the high grade tumors reveals a higher level of expression in the grade IV astrocytomas than in the grade III tumors. The role of MMP-1 in brain tumors has yet to be evaluated let alone elucidated. The research presented in this dissertation will hopefully begin a discussion on possible roles for this protein in GBMs.

1.3.2 Single Nucleotide Polymorphism

A polymorphism discovered in the MMP-1 promoter region affects the regulation of MMP-1. This section will be devoted to a literature review of the pertinent papers that have evaluated the polymorphism in various cancers and normal cells. Constance Brinckerhoff's research group was the first to identify the MMP-1 SNP[47]. Of the cell lines they routinely used for experiments, one expressed very low levels of MMP-1 mRNA and one expressed very high levels of MMP-1 mRNA and was very aggressive in culture. Analysis of the differences between these two cell lines revealed that they contained a different MMP-1 promoter. The highly expressing, aggressive melanoma cell line, A2058, contained an additional guanine base at position -1607 in

the promoter region. The cell line derived from human foreskin fibroblasts did not have this additional base at that position. The promoter that contained the additional guanine base at position -1607 was designated the 2G allele because there is a guanine base at position -1606. The promoter that did not contain the additional guanine base at position -1607, was designated the 1G allele (Figure 2). Upon further examination of the sequence around this guanine base, the group realized that this additional guanine base combined with the bases flanking it created a theoretical binding site for the ETS family of transcription factors. This intrigued Rutter and colleagues because adjacent to the additional ETS binding site was a binding site for the AP-1 family of transcription factors. Many MMP promoters, including MMP-1 are regulated by adjacent ETS and AP-1 binding sites[21]. This was mentioned in the general MMP regulation section and will be discussed more thoroughly in the MMP-1 transcriptional regulation section. Both the ETS and AP-1 transcription factor families will be discussed in greater detail in a subsequent section.

To determine if the cell line with the 2G allele was more aggressive and expressed more MMP-1 mRNA as a result of this polymorphism, the group needed to determine if this introduced ETS site was indeed *bona fide*, for example bound transcription factors, and not just theoretical. The authors conducted an electrophoretic mobility shift assay (EMSA) to determine the binding pattern to this promoter. There was no difference in the binding pattern between the probe mimicking the 1G promoter and the probe mimicking the 2G promoter. The proteins seemed to bind to the 2G probe with a higher affinity than to the 1G probe suggesting that the 2G site was a "better" binding site for the proteins. Supershift EMSAs for ETS family members were unsuccessful, however recombinant Ets-1 and cJun were able to bind to the 2G probe. The authors

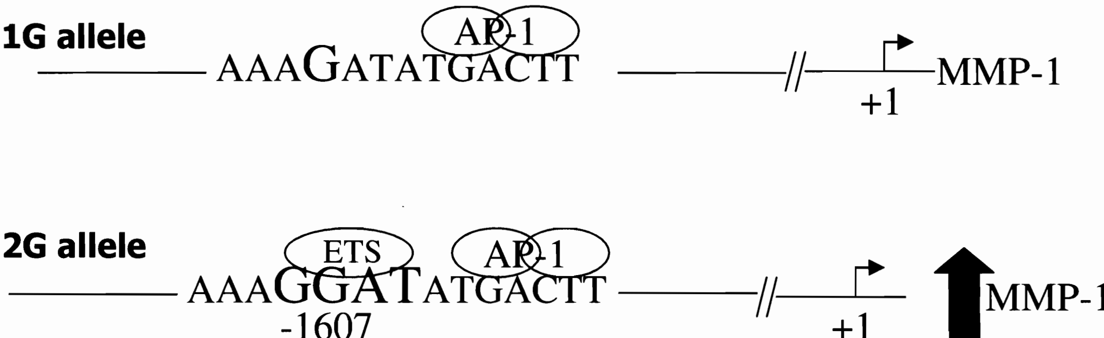
also conducted luciferase promoter assays with both promoters and discovered that the promoter construct with the 2G allele was much more active than the promoter construct containing the 1G allele. This increase in transcription from the 2G promoter could be one possible explanation for the increase in MMP-1 in the A2058 cell line.

Given this information, other research groups wanted to know if the polymorphism was important in the disease or area of their interest. Since its discovery, this polymorphism has been studied in many types of cancer and in a few non-neoplastic cell types. The remainder of this section will give a brief review of each of these studies and the findings of the authors.

Hirata *el al.*[48] evaluated the distribution of the SNP in patients with renal cell carcinoma and compared it to healthy controls. There was a statistically significant increase in the percentage of patients containing the 2G/2G genotype, that is, the patient contains two 2G alleles. Interestingly, the increase in patients with the 2G/2G genotype was found only in men; women with this disease did not have an increase in the 2G/2G genotype when compared to the control population. This group also investigated a possible loss of heterozygosity (LOH). That would occur if a patient who contained the 1G/2G genotype lost one of the two alleles and then contained either the 1G/- or -/2G genotype. LOH is not uncommon in cancer cells because of genome wide instability. In this type of cancer however, the authors did not find any evidence of LOH.

The study of endometrial carcinomas also did not reveal any LOH[49]. In the analysis of the distribution of the alleles in comparison to control populations, there was a significantly higher level of 1G/2G and 2G/2G in the patient population. MMP-1 immunohistochemistry indicated a higher level of MMP-1 expression in the cells with

Figure 2. Schematic of the single nucleotide polymorphism in the MMP-1 promoter. The presence of a guanine nucleotide at position -1607 in the MMP-1 promoter creates a binding site for the ETS family of transcription factors. The 2G allele is more transcriptionally active than the 1G allele.


1G allele
AP-1
AAAGATATGACTT
+1
MMP-1
2G allele
ETS
AP-1
AAAGGATATGACTT
-1607
+1
MMP-1

either 1G/2G or 2G/2G than the cells containing a 1G/1G genotype. The genotype however did not correlate with any clinicopathological factors.

In an analysis of LOH in 61 metastatic melanoma patients, 39% of these patients did have an LOH[50]. In this case, the patients with the 1G/2G genotype lost one of the alleles. In 83% of the cases the 2G allele was retained, indicating that the 1G allele was lost and in 17% of the cases the 1G allele was retained, indicating that the 2G allele was lost.

Chondrosarcoma is another cancer that the MMP-1 SNP has been evaluated in[51]. This group identified a chondrosarcoma cell line with a high level of MMP-1 mRNA and a cell line with low level expression of MMP-1 mRNA. Similar to Rutter's findings[47], the high expressing cell line contained a 2G/2G genotype and the low expressing cell line contained a 1G/1G genotype.

There is no significant difference in the distribution of the MMP-1 SNP in oral squamous cell carcinoma, buccal squamous cell carcinoma and oral submucous fibrosis[52]. There is an increase in the number of 2G/2G patients with non-buccal squamous cell carcinoma however. Interestingly, the controls used in this study were from Taiwan and the general population in that control group had a high percentage of 2G/2G healthy individuals. If one compares the Taiwanese patient group using the Caucasian population as a control group then it is likely that there would be a significantly different distribution in these diseases but this has not been evaluated.

Another group that used Japanese individuals for the control population also did not find any statistically significant difference between the control and Japanese patient distributions[53]. The authors did uncover a difference in behavior in cutaneous

malignant melanoma. The tumors with a 2G allele were more deeply invasive than those with a 1G allele.

There are conflicting reports about the SNP in ovarian cancer. Wenham *et al.*[54] could not find a link between either the distribution of the MMP-1 genotype in comparison with controls or in any clinical findings. This data is in conflict with the evidence presented by Kanamori *et al.*[55]. They discovered a statistically significant difference between patients with ovarian cancer and controls when the 1G/2G and 2G/2G genotypes were combined. Results from immunohistochemistry also indicate that the cells containing a 2G allele express more MMP-1 than those that do not contain the 2G allele.

The MMP-1 SNP has also been studied in lung cancer[56]. There is an increase in the 2G/2G genotype in lung cancer patients when compared to controls. This increase in the 2G/2G genotype is higher in men than in women, similar to the Hirata study[48]. The genotype of the patient was not associated with smoking status however, if a person smoked and they contain the 2G/2G MMP-1 promoter genotype they had a three fold higher risk of developing lung cancer. In moderate smokers, men had the highest risk of developing lung cancer but in heavy smokers, men and women had equal chances of developing the disease. Patients with the 2G/2G genotype also had a statistically significant reduction in the age of onset of the disease.

Ghilardi *et al.*[57] studied this polymorphism in colorectal cancer. They discovered an increase in the 2G/2G genotype in patients when compared to the control group. There was also a correlation between the 2G/2G genotype and increased metastasis.

Although there is no significant difference in the distribution of the SNP between controls and patients with breast cancer, there is evidence that the SNP contributes to lymph node metastasis[58]. Patients with a 2G/2G genotype were more likely to have lymph nodes positive for breast cancer indicating a metastatic event to the node from the breast tissue.

Cervical cancer is another example of a cancer that does not differ with respect to the distribution of the genotype relative to controls but there is a phenotypic difference in cells with the 2G allele[59]. The immunohistochemical staining of these tissues reveals similar levels of MMP-1 in both the 1G/2G and the 2G/2G cells but these are both higher than the 1G/1G containing cells. The cells with the 2G promoter are correlated with higher stages of cervical cancer implicating the SNP in the progression of the disease.

This polymorphism has been studied in non-neoplastic tissues as well as cancer tissue. Fujimoto *et al.*[60] investigated the role of the SNP in premature rupture of fetal membranes. There was a moderate but significant increase in risk for preterm premature rupture of fetal membranes in African American women with a 2G allele. This study was also the first to find a link between the SNP and ethnic status.

Wyatt *et al.* investigated the distribution of the MMP-1 SNP in human foreskin fibroblasts[61]. They found a distribution similar to the published results for the healthy population. The MMP-1 expression level did not correlate with the polymorphism however if a growth factor or stimulant was added, such as EGF, FGF or IL-1, the transcription from the 2G promoter was stimulated more than transcription from the 1G promoter. Chapter 4 will go into detail into the results of my data regarding HGF stimulation of the 1G and 2G alleles. This polymorphism has also been studied in

brain tumors[44], but this work was conducted as a part of this thesis and so is discussed in Chapter 3.

1.3.3 MMP-1 Regulation

MMP-1 is normally found in low levels in healthy adult tissues and becomes elevated during normal remodeling processes and during pathological conditions[36]. As with other MMPs, this enzyme is secreted as a proenzyme. The latency of this enzyme is maintained by the interaction of the cysteine and the zinc ion which prevents the formation of the water and zinc complex that is necessary for enzyme activation. Full length pro-MMP-1 has a molecular weight of approximately 57 kDa. This is converted to an active enzyme in one of two ways. The cysteine-zinc bond can be chemically disrupted by agents such as APMA (this is usually done in the context of *in vitro* studies) or it can be proteolytically disrupted by other enzymes[36]. Serine proteases attack pro-MMP-1 at the middle of the propeptide domain, between the Glu-Lys-Arg-Arg-Asp amino acid residues. This initial cleavage generates a short-lived 46 kDa protein that is rapidly cleaved at a Val-Met bond. This new species (43 kDa) is relatively stable but only contains about 25% of the total collagenolytic activity. Conversion to the fully active 41 kDa enzyme is carried out by other MMPs, notably MMP-3,-7,-10, at a Gln-Phe bond. Autolysis of this form of MMP-1 results in the loss of the C terminal portion of the enzyme which can no longer cleave collagen. It does retain some ability to cleave gelatin and casein however[62].

The majority of MMP-1 regulation occurs at the level of transcription. MMP-1 has a low level of basal transcription therefore the levels of steady state mRNA are often undetectable in normal adult tissues[62]. The human MMP-1 promoter contains a

TATA box at position -30, an AP-1 binding site at position -77 and an ETS binding site at position -83[36,62]. Mutation of the AP-1 site dramatically reduces the basal transcription level suggesting that this site is necessary for basal levels of MMP-1 promoter activity[62]. A promoter with both the ETS and AP-1 sites is slightly more active than a promoter containing the AP-1 site alone. This suggests that ETS proteins contribute in some way to basal transcription of the MMP-1 promoter but it is not as important as AP-1. Overexpression of Ets-1 increases the level of MMP-1 transcription[63], again suggesting a role for Ets-1 in MMP-1 basal transcription.

MMP-1 transcription can be regulated by several factors and different mechanisms are responsible in different cells and tissues. Growth factors, hormones, and cytokines are a few examples of agents capable of controlling MMP-1 promoter activity. Phorbol myristic acetate (PMA) mimics many of the signal transduction pathways and phosphorylation events stimulated by inflammatory cytokines through activation of protein kinase C. Induction of the MMP-1 promoter by PMA requires three regulatory elements: the AP-1 binding site (-77 bp) , the ETS binding site (-83 bp) and a TTCA motif present at position -109[64]. Physical stress from mechanical trauma, heat and solar radiation increase MMP-1 promoter activity. This is most likely due to activation of AP-1 protein members. This is the proposed mechanism of action for the induction of MMP-1 by cytokines as well. Another molecule that can activate MMP-1 transcription is the very molecule that it cleaves in many tissues. As type I collagen levels increase in a cell or if the cell is growing on type I collagen, MMP-1 promoter activity increases[40,65]. Lastly, as previously discussed, MMP-1 transcription can be affected by a polymorphism at position -1607.

MMP-1 transcription can be inhibited as well. TGFβ, glucocorticoid hormones, retinoids, p53 and doxorubicin can all repress MMP-1 promoter activity. Treatment with retinoic acid will inhibit MMP-1 transcription and mRNA levels[66]. Retinoic acid receptors work in one of two ways. They most often bind directly to AP-1 proteins which prevents them from binding to the DNA in the promoter, but they can also form a complex at the AP-1 binding site which will prevent AP-1 from binding to this site and activating transcription[62]. The MMP-1 promoter does not contain a consensus binding site for p53 yet this protein can also repress MMP-1 transcription. Wild type p53 down-regulates MMP-1 promoter activity in both unstimulated and stimulated cells. This repressive effect is lost if the p53 is mutated. Use of the p53 mutants commonly found in many cancers does not inhibit MMP-1 transcription[67,68]. p53 works in a similar fashion to the retinoic acid receptor in that it binds to AP-1 and thus prevents AP-1 from binding to the binding site in the promoter and activating transcription[69]. This occurs in both basal and stimulated transcription. Treatment with doxorubicin in clinically relevant doses will also repress MMP-1 mRNA and protein but this mechanism has not yet been elucidated[70,71].

Once MMP-1 mRNA is transcribed it is very unstable in resting cells. Certain agents regulate MMP-1 by increasing the mRNA stability. Naturally occurring agents such as IL-1 and EGF will increase MMP-1 mRNA stability which can then be detected by experimental means[62].

The last method to discuss related to MMP-1 regulation is enzyme inhibition. MMP-1 can be inhibited by TIMP-1 or by chelating agents such as EDTA and tetracyclines[36] but the major inhibitor of MMP-1 is α2-macroglobulin. α2-macroglobulin is composed of four subunits which have a total molecular weight of 725 kDa. This

molecule, which accounts for over 95% of MMP-1 inhibition, binds to the MMP-1 active site and irreversibly inhibits the enzyme by targeting it for clearance from the cell[62].

1.3.4 ETS Transcription Factors

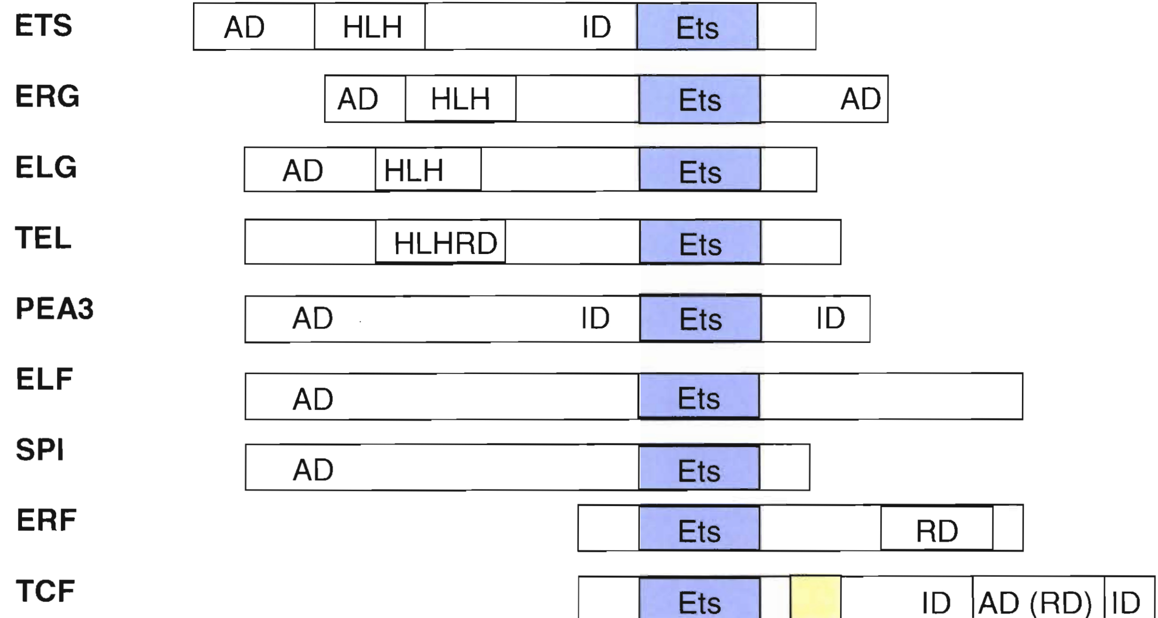
The polymorphism in the MMP-1 promoter creates an additional binding site for the ETS family of transcription factors. They are so named after the founding member, Ets-1, which was originally identified as a fusion oncogene of the avian transforming retrovirus E26. This virus induced erythroblastic and myeloblastic leukemias in chickens[72-75]. There is some discrepancy as to what the initials stand for. Certain groups say it stands for E26 transformation specific[72,73], while others say it stands for E twenty six[74,75]. Regardless of the history on the naming of the protein, there is no disagreement as to the classification, structure, function or importance of these molecules in human biology.

There are over thirty members of the ETS family of transcription factors (Table 2). They encode nuclear transcription factors that regulate gene expression. ETS transcription factors share an evolutionarily conserved ETS domain of approximately 85 amino acid residues that facilitate binding to purine rich DNA sequences with a central GGAA/T core[72-76]. This ETS binding domain is the unifying feature of the protein family. They can be further classified into subfamilies based upon structural composition (Figure 3) and similarities in the DNA binding domain[72]. DNA binding affinity is affected by two things: 1) the ETS domain and 2) flanking sequences both on the ETS protein itself and on the DNA sequence flanking the GGAA/T binding motif. In addition to directing protein-DNA interactions the ETS binding domain may also be a target for protein-protein interactions. It can have important regulatory consequences

TABLE 2. Members of the ETS family of transcription factors

Sub-family	Biological Role
Ets	extracellular matrix remodeling, multiple developmental roles
Ets-1	
Ets-2	
TCF	immediate early gene regulation, neuronal differentiation, T cell differentiation
Elk-1	
SAP-1	
SAP-2/Net	
Lin-1	
Erg	megakaryocyte differentiation
Fli-1	
Erg	
PEA3	neuronal pathfinding, sensory to motor neuron connections, muscle differentiation
PEA3	
ERM	
ER81	
GABP	muscular synapse function
GABPα	
Elf	
Elf-1	
NERF-1/-2	
MEF	
Spi	myeloid and lymphoid differentiation B cell function
PU.1	
SpiB	
SpiC	
Yan	several developmental roles
TEL	
TEL2	
Yan	
Erf	
ERF	
PE-1	

Figure 3. Schematic of the structure of the members of Ets family proteins. ETS member proteins can be divided into nine subfamilies based on structural components. Ets, DNA-binding (Ets) domain; HLH, helix–loop–helix domain (Pointed domain); AD, activation domain; ID, auto-inhibitory domain; RD, repression domain. This figure was adapted from Oikawa et al. Gene 2003 303:11-34


Subfamily
ETS
AD
HLH
ID
Ets
ERG
AD
HLH
Ets
AD
ELG
AD
HLH
Ets
TEL
HLHRD
Ets
PEA3
AD
ID
Ets
ID
ELF
AD
Ets
SPI
AD
Ets
ERF
Ets
RD
TCF
Ets
ID
AD (RD)
ID

such as regulating DNA binding and transcriptional[75]. The evolutionarily conserved pointed domain is responsible for the formation of a helix loop helix which is important in protein-protein interactions[72]. ETS proteins do not seem to associate as homo or heterodimers with other ETS family members but can form complexes with transcription factors in unrelated families. Alone they have weak transactivating abilities therefore they require co-factors for optimal activity[73,77,78]. Co-factors known to interact with ETS proteins include: AP-1, AML1, LEF-1, Sp1, cMyb, Pax5, NFκB, Stat-5 and Maf-B. These are the most common binding partners but this is not an all inclusive list.

ETS proteins belong to the winged helix loop helix family of binding proteins. This is a subtype of a DNA binding motif that consists of two α helices separated by a tight turn. This binding motif contains an extended loop between two β strands (the wing). The main protein-DNA contacts are formed by the residues that are located in the third α helix and in the wing between β strands three and four. DNA also contacts the protein in a loop between α helices two and three[72,74,75].

ETS proteins can activate or repress transcription in cooperation with co-factors and regulate a variety of cellular functions including growth, apoptosis, development, and oncogenic transformation. They can regulate growth factors and growth factor receptors, cell cycle related genes and the differentiation of many tissues including hematopoietic cells, vascular endothelial cells, myogenic and osteogenic as well as neuronal differentiation[72]. Embryonic expression of Ets-1 is primarily associated within sites of active remodeling of the ECM. It is expressed in astrocytes and is upregulated during retinoic acid induced neuronal differentiation[72,74]. Ets-2 is expressed strongly in the brain during the late stages of CNS development and is maintained in the

cerebellum, hippocampus, and cerebral cortex of adult mammals[75]. Members of the PEA3 subfamily also appear to be involved in the development of the CNS. PEA3 is expressed in specific bundles of motor neurons that innervate limb muscles and appear in afferent sensory neurons of these same muscles. Another member of the PEA3 subfamily, ETV1 is expressed in distinct neuronal subsets of the developing spinal cord to control a late step in the establishment of functional sensory to motor neuron connections[72,74].

Of particular interest to this discussion is the ability of ETS proteins to regulate genes known to be involved in tumor biology. This includes genes involved in tumor invasion, metastasis and angiogenesis[79,80]. ETS members are expressed in vascular endothelial cells and the expression of VEGF, bFGF and TNFα all induce expression of the Ets-1 gene. The role of ETS proteins in tumor invasion is largely related to the transcriptional activation of enzymes that are involved in ECM degradation, namely serine proteases, MMPs and TIMPs[78]. The expression patterns generally correlate with the tumor grade and invasion and metastasis[72]. Upregulation of Ets-1 expression has been documented in many types of human tumors. Immunohistochemistry and in situ analysis of mRNA of a variety of human tumors indicates that Ets-1 and matrix degrading enzymes are coexpressed in invasive tumors. In non-invasive and benign tumors, Ets-1 is rarely detected. Antisense oligonucletotides transfected into glioma cell lines that inhibited the expression of both Ets-1 and uPa blocked not only glioma cell migration but also invasion of the glioma cells. The use of a mutant Ets-1 protein lacking the activation domain leads to similar phenotypic changes in glioma cells[78].

Post translational modifications, including phosphorylation, often change their ability to bind to DNA, activate transcription, and the ability to associate with cellular

partners. They can also affect the subcellular location of the protein and affect the stability of the protein[72,75]. Many ETS proteins can be phosphorylated as a result of activation of the MAP kinase signaling pathway[79]. This pathway will be discussed in detail in the signal transduction section.

1.3.5 AP-1 Transcription Factors

Activating Protein-1 (AP-1) is a collective term referring to dimeric transcription factors composed of Jun, Fos, activating transcription factor (ATF) or musculoaponeurotic fibrosarcoma (Maf) subunits that bind to a common DNA site[81] (Table 3). They are classified as basic leucine zipper (bZIP) proteins because they dimerize through a heptad repeat of leucine residues and contain a basic domain for interaction with the DNA backbone[82,83]. Fos and Jun were first identified as the viral oncoproteins, vFos and vJun in the Finkel-Biskik-Jinkins osteosarcoma and avian sarcoma virus 17, respectively[83]. They bind DNA recognition elements known as the TPA recognition element (TRE) so named because of their ability to mediate transcription in response to the phorbol ester TPA[84].

AP-1 proteins bind as a dimer which can be composed of either the same protein (homodimer) or two different proteins (heterodimer). Jun, as well as ATF, proteins can form stable homodimers while Fos and Maf proteins cannot. Jun proteins can form stable heterodimers with Fos and ATF. Fos proteins can form heterodimers with all of the Maf proteins but Jun can only form heterodimers with vMaf and cMaf. Different dimer combinations can recognize different sequence elements in the promoter and enhancer regions of DNA[81,83]. A Jun/Fos complex binds more tightly to DNA than either Jun or Fos alone[82].

Table 3. Members of the AP-1 family

Subfamily	Name
JUN	vJun
	cJun
	JunB
	JunD
FOS	vFos
	cFos
	FosB
	Fra1
	Fra2
ATF	ATF2
	ATF3/LRF1
	B-ATF
MAF	vMaf
	cMaf
	MafB
	MafF
	MafG
	MafK

There are four main levels of regulation of the AP-1 transcription factor family: 1) changes in gene transcription and mRNA turnover 2) protein turnover 3) post translational modification and 4) interactions with other transcription factors that can either synergize or inhibit AP-1 activity[83,85]. AP-1 activity can be induced by growth factors, cytokines, neurotransmitters, polypeptide hormones, cell-matrix interactions, bacterial and viral infections and a variety of physical and chemical stresses[85]. The majority of these inducers will stimulate the mitogen activated protein (MAP) kinase pathway ultimately leading to phosphorylation of AP-1 proteins. This pathway will be discussed in detail in a subsequent section.

AP-1 proteins function in a variety of cellular processes. They are mostly known for their role in cellular proliferation and oncogenic transformation but newer roles are becoming established for this protein family. They include regulation of apoptosis, both pro and anti apoptotic, invasion and angiogenesis[81,86,87]. AP-1 proteins in concert with ETS transcription factors are well known mediators of MMP family members and as such can regulate invasion in the correct cellular environment.

1.4 Hepatocyte Growth Factor/Scatter Factor

1.4.1 Overview

This section will focus on the structure and function of both hepatocyte growth factor/scatter (HGF/SF) and its receptor, the proto-oncogene, Met. The following sections will give a brief review of the signal transduction pathways that become activated following HGF/SF binding to the Met receptor and then the introduction chapter will end with a review of the relevant information in the literature regarding HGF/SF and gliomas.

Met was first identified as an oncogene in the 1980s. The met gene was known to encode a tyrosine kinase receptor but at that time the ligand for the receptor was unknown. The ligand was discovered by two different groups working independently. One discovered a potent motility factor and named it scatter factor and the other described a mitogenic factor for hepatocytes and named it hepatocyte growth factor[88]. The two molecules turned out to be identical therefore both names were kept and the molecule is referred to as HGF/SF.

HGF/SF is a large multidomain protein that is similar to plasminogen. It consists of six domains: one amino terminal domain, four kringle domains, and one serine proteinase homology (SPH) domain (Figure 4). It is synthesized as pro HGF/SF, a single chain that is inactive until it is proteolytically cleaved. Cleavage occurs at a trypsin like site located after the K4 domain producing a disulfide linked α and β chain heterodimer[88]. Serine proteases are the enzymes responsible for the proteolytic activation of HGF/SF.

The main receptor binding site of HGF/SF is located in a domain known as NK1, a combination of the N terminal domain and the K1 domains. NK1 can bind to two met molecules aiding in receptor dimerization. The met receptor is also a disulfide linked heterodimer, and is also produced in an inactive form. Cleavage of a furin site located between amino acid residues 307 and 308 results in the mature form of the receptor[88]. It is composed of two chains, the α chain and the β chain (Figure 5). The α chain is 50 kDa in size and is solely extracellular. The β chain is 145kDa and is a transmembrane domain. The extracellular moiety of the β chain contains two elements: a sema domain (500 amino acids) known to function in protein-protein interactions and a cysteine rich motif (80 amino acids) called the met related

Figure 4. Schematic of hepatocyte growth factor/scatter factor. The domain structure of hepatocyte growth factor/scatter factor. N, amino-terminal domain; K1–K4 kringle domains 1–4; SPH, serine proteinase homology domain. α and β refer to the subunits of the ligand that are present after proteolytic cleavage. This figure was adapted from Birchmeier et al Nature Reviews Molecular Cell Biology 2004 4:915-925.

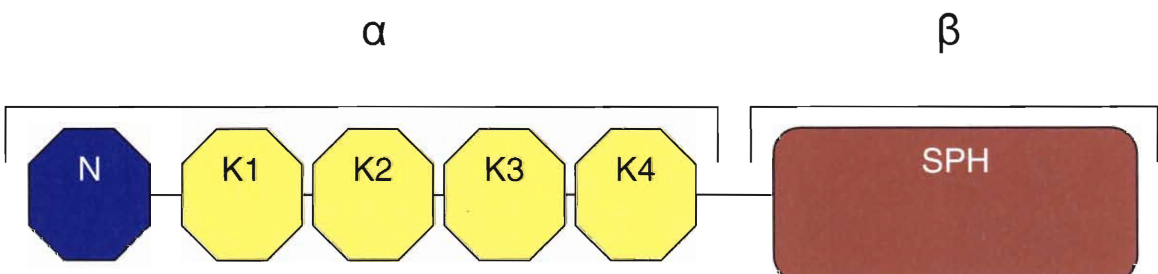
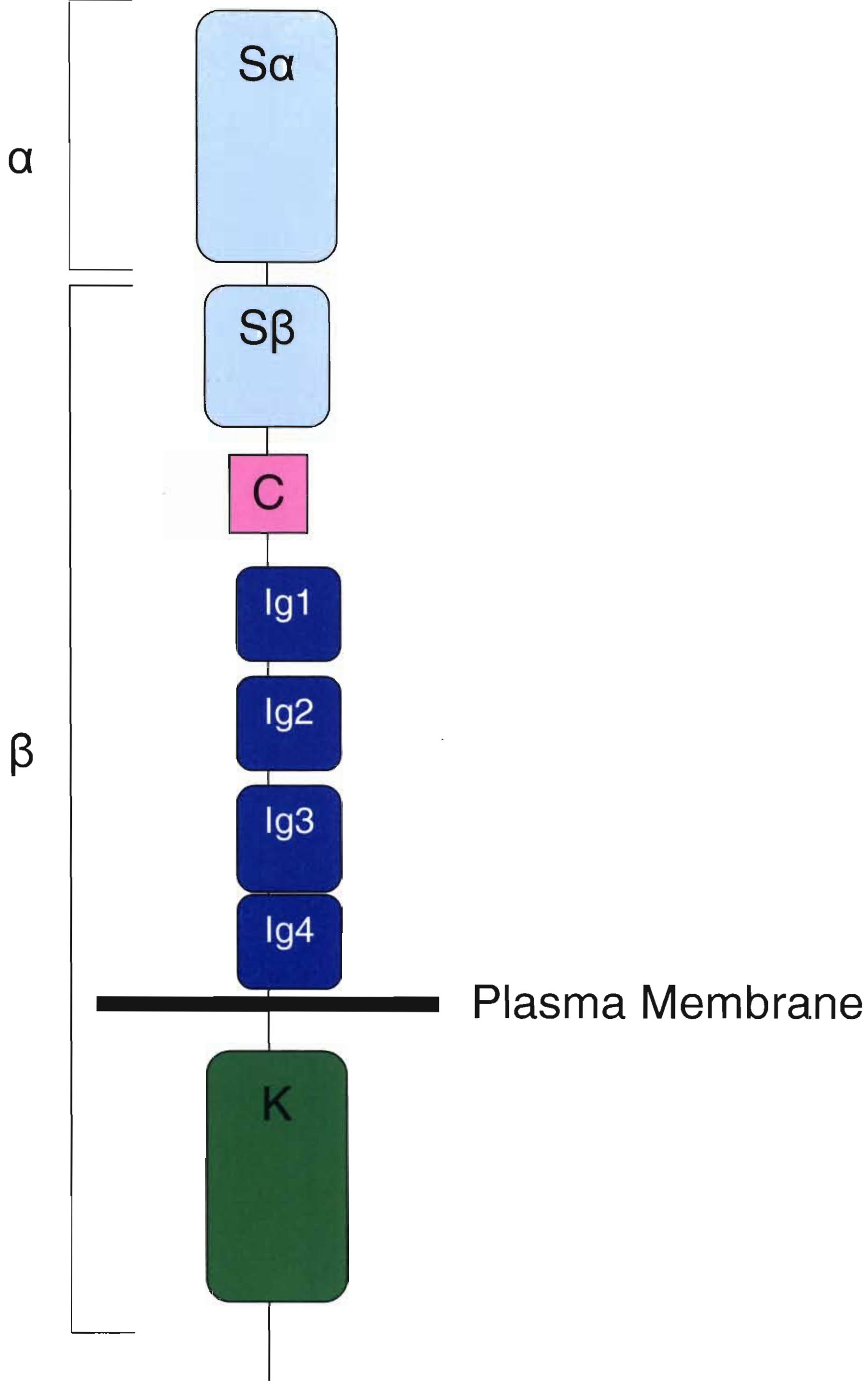

α
β
N
K1
K2
K3
K4
SPH

Figure 5. Schematic of the Met receptor tyrosine kinase. S, sema domain; C, cysteine-rich domain; Ig, immunoglobulin domain; K, kinase domain; α and β refer to the subunits of the receptor that are present after proteolytic cleavage. This figure was adapted from Birchmeier et al Nature Reviews Molecular Cell Biology 2003 4:915-925.


α
Sα
Sβ
β
C
Ig1
Ig2
Ig3
Ig4
Plasma Membrane
K

sequence[89]. The cysteine rich sequence is followed by four repeats of an unusual type of immunoglobulin domain. The function of this domain is not completely clear but it is thought that it might keep the correct orientation for ligand binding with the α domain. The intracellular portion of the β chain contains three functional domains: 1) the juxtamembrane domain which can serve as an inhibitory domain if phosphorylated 2) the tyrosine kinase autocatalytic domain which is induced by receptor transphosphorylation and 3) a C-terminal tail with a docking site for recruitment of downstream signaling molecules[88,89]. Structural studies have identified the α domain and the first 212 amino acid residues of the the β chain as sufficient for HGF/SF binding. The structural features described above are sufficient to categorize this receptor as a member of the scatter factor receptor family. Other members of this family include Ron and the chicken homologue, Sea[88,89].

Genetic analysis of HGF/SF and Met indicate physiological functions in development and wound repair. The genes for both HGF/SF and Met are expressed in close proximity. This indicates that HGF/SF has a limited capacity to diffuse over a large distance *in vivo*. The expression of HGF/SF and Met is upregulated in several injured tissues such as the liver, the kidney and the heart[88]. HGF/SF is a potent liver mitogen and stimulates the proliferation of hepatocytes, increases the overall liver size and enhances liver regeneration. This suggests that HGF/SF is part of a general defensive response to tissue damage.

HGF/SF and Met contribute to the establishment of tissue patterning and the onset and maintenance of normal organ architecture[89]. Muscle development, nervous system formation, hematopoietic differentiation, bone remodeling and angiogenesis are the main processes dependent on HGF/SF and Met. They provide essential signals for

survival and proliferation of both hepatocytes and placental trophoblasts during development. Mice lacking Met have impaired or reduced survival of sensory or sympathetic neurons and an impaired outgrowth of particular motor nerves. One reason for this could be limited nutrient and oxygen supply due to the placental defect[88]. Another role for HGF/SF and Met in development includes the control of the growth and survival of epithelial cells and the migration of myogenic precursor cells. Animals without HGF/SF or Met genes are lacking all of the muscle groups that are derived from migrating progenitor cells[88].

The release of muscle progenitor cells resembles the process that occurs during the progression of carcinomas[88]. Invasive cells are released from the primary tumor in a process called epithelial mesenchymal transition (EMT). During EMT in normal cells, epithelial cells adjacent to a wound site detach from each other and change shape. They then rapidly migrate towards the wounded area where they invade the injury site, proliferate and reconstitute the epithelial layer[89]. This occurs in carcinomas during the process of metastasis or formation of a secondary tumor. During the migration of the cells, either normal or tumor cells, survival in the bloodstream is helped by Met. It protects them from apoptosis and allows the cells to transiently grow in an anchorage independent manner, until the migration to the secondary site is complete. This function of Met is beneficial in wound repair but it is manipulating the system by using this for metastasis.

Activation of Met in cancer occurs most often in one of two ways, either through autocrine or paracrine signaling mechanisms[88,89]. Autocrine signaling occurs in GBMs. It is when the cell secretes both the ligand (HGF/SF) and the receptor (Met). In paracrine signaling the ligand is secreted by a cell other than the cell secreting the

receptor. For example, the tumor cell will secrete the receptor but the ligand is secreted by cells in the stroma.

The dysregulation of Met and HGF/SF is a feature of most human malignancies and correlates with a poor prognosis. It can cause proliferation, invasion, angiogenesis and prevent apoptosis in tumor cells. Met overexpression is the most frequent alteration in human tumors[89]. Under physiological conditions, Met expression is a transient event however in tumors it is often constitutively active. Constitutively active Met causes neoplastic cells to separate from the tumor mass, invade the basement membrane and form tumors elsewhere in the body. Met can be activated even in the absence of HGF/SF in tumors. This can be caused by Met gene amplication, enhanced Met transcription or in a newly discovered mechanism, through hypoxia activated transcription[89]. The Met receptor can also be activated in an HGF/SF independent manner by transactivation by other membrane receptors such as Ron, and the EGF family member receptors FAS and B plexins.

The downregulation of Met and HGF/SF expression in human tumor cells decreases their tumorigenic potential. Due to this fact, many investigators are studying these two proteins and their interaction as one potential therapeutic against many types of cancers.

1.4.2 Signal Transduction

This section will be a brief review of one of the ways MMP-1 gene expression can be activated: the signaling pathway that begins when HGF/SF interacts with its receptor. The binding of HGF/SF to the Met receptor causes receptor dimerization and autophosphorylation of the receptor. This leads to the recruitment and activation of

many intracellular proteins such as Gab1, Grb2 and Shc. These intracellular proteins are responsible for the activation of the Ras protein (Figure 6). Ras directly associates with kinases and initiates the MAP kinase cascade[90,91].

The MAP kinase cascade features a core triple kinase cascade. Ras forms a complex with the MAP kinase kinase kinase (MAPKKK) Raf which results in the translocation of Raf to the cell surface. This translocation is necessary for the activation of Raf's kinase activity. Activated Raf phosphorylates the MAP kinase kinase (MAPKK) MEK which activates the MAP kinase ERK (Figure 7 left column). There are four main groups of MAP kinases: extracellular signal related kinase (ERK), cJun N-terminal kinase (JNK), p38 and extracellular signal related kinase-5 (ERK5, also called Big MAP kinase-1 (BMK1)). ERK is mainly activated by mitogenic stimuli such as growth factors and hormones while JNK and p38 are mainly activated through stress stimuli. ERK5 can be activated by both growth factors and stress[92].

Once activated, the MAP kinases can directly phosphorylate transcription factors at the phosphoacceptor site: a serine/threonine residue followed by a proline residue. Phosphorylation of a transcription factor regulates its activity in many ways. It can affect the intracellular location of the protein. For example it can alter the structure of the nuclear localization sequence causing the movement in or out of the nucleus. It can affect the protein levels of the transcription factor or the ability of the protein to bind to DNA promoter elements. Lastly, it can affect the interactions with other regulatory proteins[90,93].

Figure 6. Schematic of the met signaling pathway. Activation of Met results in the recruitment of scaffolding proteins like Gab1 (growth-factor-receptor-bound protein 2 (Grb2)-associated binder 1) and Grb2, which activate Shp2, Ras and ERK/MAPK. α and β refer to the subunits of the receptor that are present after proteolytic cleavage. This figure was adapted from Birchmeier et al Nature Reviews Molecular Cell Biology 2003 4:915-925.

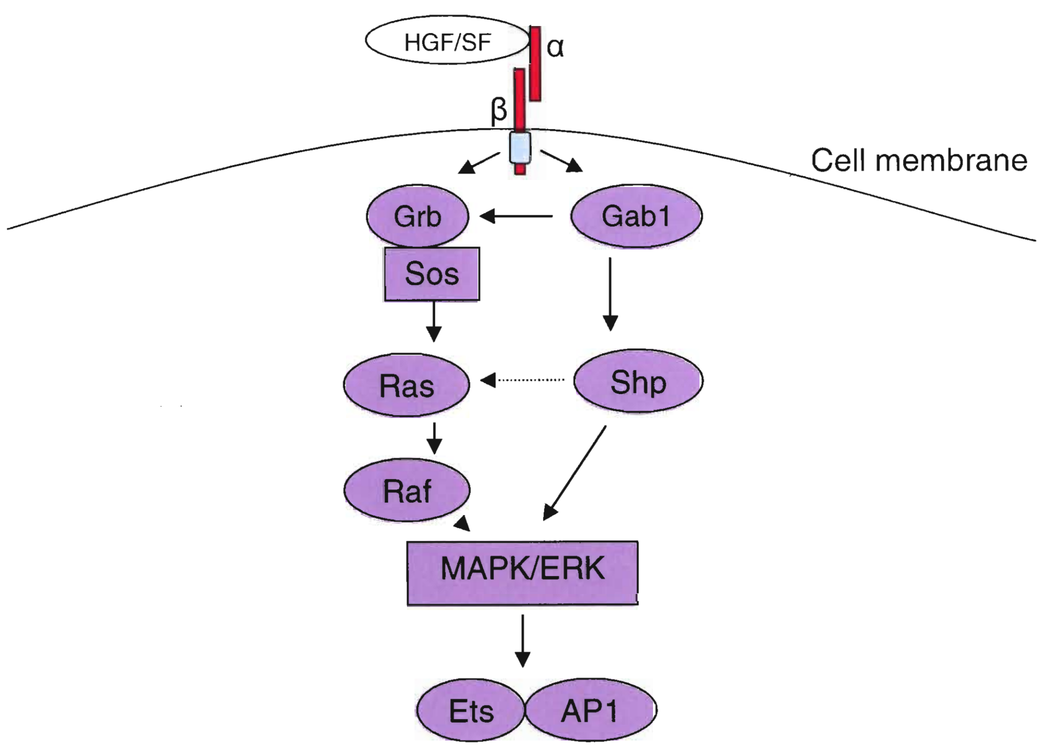
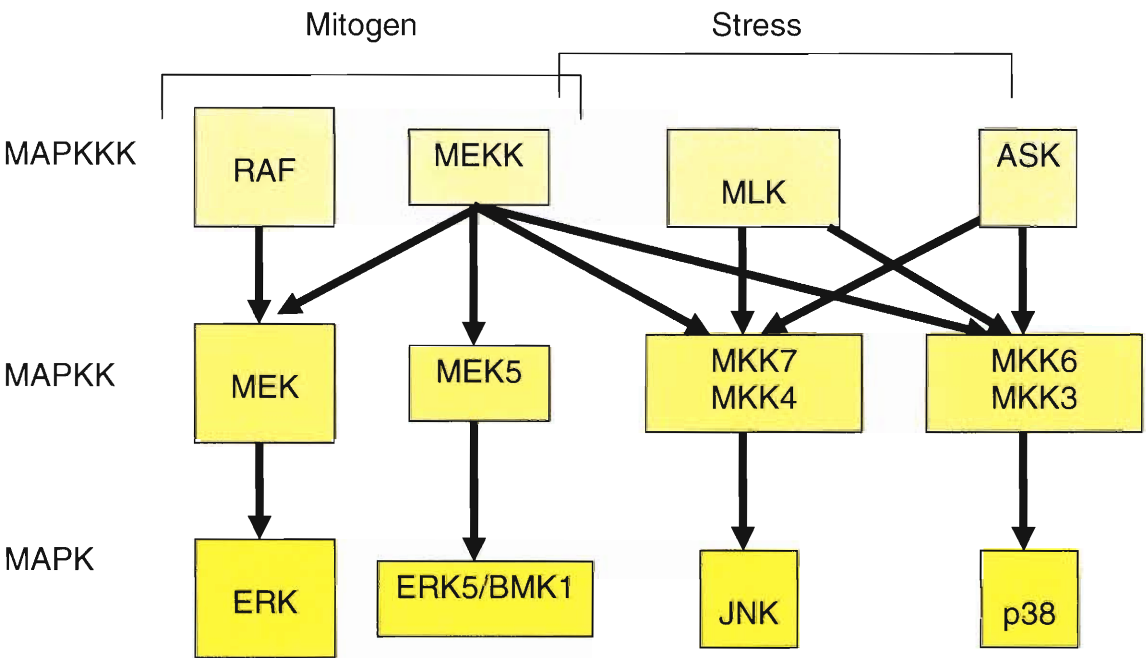

HGF/SF
α
β
Cell membrane
Grb
Gab1
Sos
Ras
Shp
Raf
MAPK/ERK
Ets
AP1

Figure 7. Organization of mammalian MAPK cascade. MAPK cascades feature a core triple kinase module consisting of MAPKKKs, MAPKKs and MAPKs. There are a number of MAPKKK families and individual MAPKKKs are often components of more than one MAPK cascade. MAPKKs selectively target a particular MAPK. A potential exception is MKK4 which has been reported to activate p38 (dashed line) in addition to its major target JNK. MAPKs can phosphorylate transcriptional targets directly or this can occur via the indicated downstream protein kinases. This figure has been adapted from Yang et al. Gene 2003 320:3-21



Mitogen
Stress
MAPKKK
RAF
MEKK
MLK
ASK
MAPKK
MEK
MEK5
MKK7
MKK4
MKK6
MKK3
MAPK
ERK
ERK5/BMK1
JNK
p38

In order for the binding of HGF/SF to the met receptor at the cell surface to have an impact on the proteins in the nucleus, that signal must travel from the cell surface to the nucleus. This is accomplished by passing the signal through the proteins just described, ultimately ending in the activation of ERK. When activated, ERK translocates into the nucleus thereby activating transcription factors located in the nucleus and completing the transduction of the signal from the cell surface.

Once it is in the nucleus, ERK can activate the ETS family of transcription factors and upregulate MMP-1 transcription[94] through phosphorylation of threonine 38 in the N terminus of the protein[91]. The MMP-1 promoter contains a Ras responsive element (RRE) that consists of a combined binding site for ETS and AP-1 family members. It is through this element that HGF/SF exerts its control on MMP-1 promoter activity. An RRE is found in the MMP-1 promoter at position -77 and if mutated, blunts the response of HGF/SF on MMP-1 promoter activity[94].

HGF/SF can also upregulate the mRNA and protein levels of MMP-1 in both hepatocytes[95,96] and keratinocytes[97] however these increases have not been linked to the MAP kinase pathway. It is likely that the mechanism described above is responsible for the upregulation but this has not been studied in these tissues to date.

1.4.3 Hepatocyte Growth Factor/Scatter Factor and Gliomas

There have been few studies looking at the relationship between HGF/SF and high grade gliomas. Most of the studies linking expression levels and high grade gliomas have been conducted on primary tumor tissue and the studies linking HGF/SF and behavioral changes have used glioma cell lines. Arrieta *et al.*[98] analyzed tumors from patients with either a grade III or a grade IV astrocytoma. They discovered an

inverse relationship between HGF/SF expression and patient survival. They also noted that the grade III tumors contained less HGF/SF than did the grade IV tumors. Koochekpour *et al.*[99] also looked at the levels of HGF/SF in high grade gliomas. They conducted immunostaining on 32 primary human tumors for Met and HGF/SF. All of the tumors were positive for Met expression and 72% of the tumors expressed HGF/SF. The expression of HGF/SF and Met corresponded to the hypercellular and infiltrative areas of the tumors. A third study analyzing the expression levels of Met in high grade gliomas using immunostaining found 77% of the tumors to be positive for Met expression[100].

HGF/SF addition to glioma cell lines in culture stimulates motility and proliferation[99,101]. It can also increase Met receptor levels in the glioma cell lines U373 and T98. Analysis of the downstream effects of this increased Met expression indicates an increase in the protein levels of AP1 family members cJun and cFos[102]. Data accumulated in the course of this thesis project is in agreement with this study and will be discussed in Chapter 4.

There is one study investigating the relationship between HGF/SF and gliomas in an animal model[103]. Abounader and colleagues established animal xenografts from U87 glioma cells and treated the mice with HGF/SF inhibitors. Upon removal of the tumor from the animal the investigators discovered that HGF/SF inhibition prevented glioma growth and angiogenesis and promoted apoptosis.

It is known that HGF/SF increases MMP-1 levels through the activation of Ets-1 and it is known that HGF/SF correlates with a poor prognosis in gliomas however there are no papers linking the increase of MMP-1 through HGF/SF-Ets-1 signaling in

gliomas. The second half of this thesis project was devoted to that and will be discussed in Chapter 4.

Chapter 2

Materials and Methods

2.1 Primary Human Samples

Tumor samples were obtained from 81 patients who underwent surgery as part of the treatment for glioblastoma. Tumor tissue obtained at time of craniotomy was dissected free of adventitial material, snap-frozen in liquid nitrogen, and maintained in a tissue bank at –80°C in accordance with an approved protocol from our Institutional Review Board (IRB #3031). Each sample was viewed by a neuropathologist to confirm the diagnosis of glioblastoma multiforme. Genomic DNA was extracted following the manufacturer's protocol for the QiaAmp DNA Mini Kit (Qiagen). Briefly, one piece of tissue was removed from the tumor bank and digested with buffers (doubled the amount suggested by the kit) from the kit overnight at 56 °C. The following morning the protocol was continued (again buffer amounts were doubled) until the DNA was collected. The genomic DNA was quantified by spectrophotometry at the wavelengths of 260 and 280. The concentration was determined by the absorbance at 260 while the quality of the DNA was calculated from the ratio of the absorbance at 260 divided by the absorbance at 280.

Fifty-seven healthy volunteers were recruited to donate DNA to use as a control. The subject was asked if he/she had eaten 30 minutes previous to the procedure. If he/she had not, the subject was asked to sign the consent form corresponding to IRB #2079. After signing the consent form, the subject was given a cotton swab to swipe the inside of the cheek six to ten times. The swab was placed in a 1.5 mL eppendorf tube and stored at room temperature until the time of the extraction. The DNA was extracted using the QiaAmp DNA Mini Kit. The protocol was similar to the tumor DNA extraction described above except that the swabs were placed in PBS and buffers supplied with the kit to remove the DNA from the swab. The

kit instructions were then followed as above. The samples were quantified in the same manner as the tumor samples.

2.2 Genotyping

The genotype of DNA samples from the 81 patients and 57 volunteers and the DNA extracted from gliomas cell lines was determined by following the protocol previously published[56]. In this protocol, genomic DNA is amplified by PCR with a pair of primers directed at the MMP-1 promoter sequence. PCR primer sequences were as follows: forward 5'- TGACTTTTAAAACATAGTCTATGTTCA-3'; and reverse 5'-TCTTGGATTGATTGAGATAAGTCATAGC-3'. The reverse primer was modified to incorporate an *Alu I* recognition site (AGCT). The 1G allele retains this recognition sequence, whereas the presence of the additional guanine nucleotide in the 2G allele disrupts this recognition sequence. The PCR product was digested overnight at 37°C with one unit of *Alu I* (New England Biolabs), 1x reaction buffer and sterile water to a total volume of 30 µL. Digestion products were separated on a 4% agarose gel for 1 hour at 100 V and visualized with ethidium bromide. The 1G homozygous samples (1G/1G) were represented by bands at 241 and 28 bp, 2G homozygous samples (2G/2G) were represented by a single band at 269 bp and the heterozygous samples (1G/2G) were represented by bands at 269, 241 and 28 bp.

2.3 Sequencing

The genotype of the gliomas, normal tissue and gliomas cell lines was confirmed by direct DNA sequencing.. PCR was performed on a subset of the samples following the protocol previously described by Hirata *et al.*[48]. PCR primer sequences were as follows: forward 5'-CAGTGGCAAGTGTTCTTTGG-3' and reverse 5'-

CTCCCACCTTTC-CCACTGTA-3'. The PCR products were sent to the University of Iowa Sequencing Facility.

2.4 Nuclear Extraction

This protocol followed a method that was previously published[104]. Glioma cells were plated at a density of 2 x 10^6 cells in 10% DMEM on100mm dishes. When cells were ready to harvest, the media was removed, cells were washed twice with ice cold PBS and scraped into 1.5 mL ice cold 1x PBS. The suspension was transferred into a 1.5 mL microcentrifuge tube and cells were pelleted through centrifugation at maximum speed in a table top centrifuge for 10 minutes at 4˚C. The supernatant was removed and pellets were resuspended in 400 µL Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, inhibitors) by flicking the side of the tube after the buffer was added. Tubes were place on ice for 10 minutes, vortexed for 10 seconds and then centrifuged for 10 seconds at room temperature, maximum speed in a table top centrifuge. The supernatant was discarded as the cytoplasmic portion and the pellet of intact nuclei was kept and resuspended in 60µL (less if want it more concentrated) of ice cold Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, inhibitors). Samples were incubated on ice for 20 minutes then centrifuged for 2 minutes at top speed in 4˚C. The pellet was discarded and the supernatant, which contains the nuclear proteins was either used immediately or stored at -80˚C in aliquots. For binding assays, samples were dialyzed on a 0.025 µ filter (Millipore Corp) floating on dialysis buffer (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, inhibitors) for one hour. Samples were quantified using the Coomassie Plus Protein Assay Kit (Pierce)

2.5 Electrophoretic Mobility Shift Assay

10 µg of nuclear extract was incubated for 20 minutes at room temperature with 9 pmol of ^{32}P end-labeled double stranded oligonucleotide mimicking the 2G MMP-1 promoter (5'-TAGAAAGGATATGAC-TTATCTCA-3') in 20µL binding buffer (1µg salmon sperm, 5% Ficoll, 5mM $MgCl_2$) For competition assays 90pmol (10 fold excess) of unlabeled double stranded oligonucleotide encompassing either the Ets-1 binding site (5'-GGAGG-AGGGCTGCTTGAGGAAGTATAAGAAT-3') or the NFkB binding site (5'-AGTTGAG-GGGACTTTCCCAGGC-3') was added to the binding buffer and labeled 2G probe. DNA-protein complexes were separated by electrophoresis at 4˚C through a 6% native polyacrylamide gel for 2 hours at 210 volts, dried for one hour at 80˚C in a vacuum dryer and exposed to autoradiography film overnight.

2.6 Transient Transfection and Luciferase Assay

Glioma cells were plated in triplicate at a density of 0.5 x 10^6 cells in a 6 well plate in 10% DMEM minus antibiotics. The cells were transfected with either the 1G or 2G full length MMP-1 promoter construct, obtained from Dr. Constance Brinckerhoff, 24 hours after plating. 2µL of Lipofectamine 2000 (Invitrogen) was added to 48 µL serum free media for a total volume of 50 µL per well. This tube was incubated at room temperature for 5 minutes. In a separate tube, 1 µg of promoter DNA and 0.1 µg of Renilla DNA was added to serum free media for a total volume of 50 µL per well. The Lipofectamine 2000:media tube was added to the DNA:media tube and incubated for 20 minutes at room temperature. During this incubation, the DMEM minus antibiotics was removed from the wells, the wells were washed with warm 1x PBS and 2 mL of serum free media was added to each well. At the end of the incubation, 100 µL of the Lipofectamine 2000:DNA complex was added to each well and the plates

were incubated at 37˚C for 4 hours. The media was then removed and replaced with 10% DMEM minus antibiotics. If the cells received growth factor treatment, the 10% DMEM minus antibiotic media was removed after six hours, cells were washed with warm 1x PBS and serum free media was added to starve the cells from serum overnight, prior to growth factor treatment.

The Dual Luciferase Kit (Promega) was used to collect the samples. At the time of collection, the media was removed, the wells were washed with room temperature 1x PBS and 500 µL of 1x Passive Lysis Buffer was added to each well. Plates were incubated at room temperature for 15 minutes on a platform shaker set to slow speed. The cells were scraped to the bottom of the well and transferred to a 1.5 mL eppendorf tube and frozen at -20˚C overnight. To measure the luciferase activity in each sample, the samples were thawed and 20 µL was pipetted into a white 96 well plate with a white bottom (Costar). The plate was inserted into the Lumi Star (BMG) Luminometer and 100µL LAR II reagent was injected into each well to measure the luminescence from the MMP-1 promoter constructs. 100µL Stop & Glo was injected into each well to measure the luminescence from the Renilla. Triplicates were averaged and the MMP-1 luminescence was divided by the Renilla luminescence and plotted as relative luciferase units.

2.7 RNA Extraction and Real Time PCR

Total RNA was extracted from tumor tissue and glioma cell lines following the TRIzol (Invitrogen) method. Tumor tissue was crushed with an autoclaved and pre-cooled mortar and pestle on dry ice into a fine powder and transferred to a 1.5 mL eppendorf tube with a spatula cooled to -80˚C. 1 mL of TRIzol was added directly to the eppendorf tube containing the tumor tissue and the tubes were incubated at room

temperature for 5 minutes. For RNA extraction of the glioma cell lines, cells were plated at a density of 2 x 10^6 in 10 % DMEM on 100 mm dishes. At the time of the collection, the media was removed, the plates were washed with room temperature 1x PBS and 1 mL of TRIzol was added directly to the plates for 5 minutes at room temperature and then transferred to a 1.5 mL eppendorf tube. 200 µL of chloroform was added to each tube, shaken vigorously for 15 seconds, incubated at room temperature for 3 minutes and then centrifuged at 12,000g for 15 minutes at 4˚C. The aqueous phase (upper phase) was then transferred to a fresh tube and 500 µL of isopropyl alcohol was added to each tube. The tube was inverted several times to completely mix the alcohol with the aqueous phase, incubated at room temperature for 10 minutes and spun for 10 minutes at 12,000g at 4˚C. The supernatant was discarded and 1 mL of 75% ethanol was added. The tube was then vortexed and centrifuged for 5 minutes at 4˚C for 7,500g. The supernatant was again discarded, the pellet was allowed to air dry for 8 minutes and 100 µL of DEPC water was used to resuspend the pellet. The sample was incubated for 10 minutes in a 55˚C water bath. The RNA is quantified by spectrophotometry as described above. Prior to real time PCR the RNA was treated with DNase (Promega). 1 µg of RNA was added to a 0.65 mL eppendorf tube with 1 µL RQ1 10x buffer and 1µL RQ1 RNase free DNase in a total volume of 10 µL. The samples were incubated for 30 minutes in a 37˚C water bath. 1 µL of Stop Solution (20 mM EGTA) was added and the samples were incubated at 65˚C for 10 minutes. Samples were sent to the Molecular Core of the Virginia Commonwealth University-Massey Cancer Center Nucleic Acids Research Facilities for Real Time PCR analysis of mRNA levels using the ABI 7900 Sequence Detection System.

2.8 Protein Extraction

Total protein from tumor tissue and glioma cell lines was extracted using two different methods. Protein was extracted from tumor tissue using Tissue Protein Extraction Reagent (T-PER, Pierce). T-PER was made by adding 150 mM NaCl to M-PER (Pierce) plus inhibitors. Tumor tissue was removed from the tumor bank, weighed and ground to a fine powder form in a mortar and pestle on dry ice and transferred to a 1.5 mL eppendorf tube using a spatula cooled to -80˚C. 2 mL of T-PER was added to the tube for each 100 mg of tumor tissue. The samples were incubated for 5 minutes at room temperature and each minute during the incubation, the samples were vortexed. Samples were then centrifuged at 10,000 rpm for 5 minutes in 4˚C to pellet the debris. The supernatant was collected, transferred to a fresh pre-cooled tube and quantified using the Coomassie Plus Protein Assay Kit (Pierce). Samples were aliquoted and stored in -80˚C until use.

Protein was extracted from glioma cell lines using RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% SDS, 1% deoxycholate). Cells were plated at a density of 2×10^6 per 100 mm dish in 10% DMEM. At the time of collection, the media was removed, cells were washed in ice cold 1x PBS and 800 µL of RIPA was added directly to the plate on ice for 5 minutes. During the incubation, the plate was rocked to allow the RIPA to come in contact with the entire surface of the plate. The cells were scraped to the bottom of the plate, transferred to a pre-cooled 1.5 mL eppendorf tube and centrifuged for 20 minutes at maximum speed in 4˚C. The supernatant was transferred to a new pre-cooled tube, quantified using the Bio-Rad DC Protein Assay Kit, divided into aliquots and stored in -80˚C.

2.9 ELISA

Wells (ELISA plate, Fisher) were coated with 100 ng of capture antibody (CLMABMMP1, Cedar Lane). The plate was covered in aluminum foil and incubated overnight in 4˚C. The following morning excess antibody was removed from the wells and the plate was tapped on a paper towel to remove excess liquid. Plate was blocked with 300 µL of blocking buffer (PBS/0.5% BSA) for two hours in 4˚C. Blocking buffer was removed from the wells and the plate was tapped on a paper towel to remove excess liquid. The plate was washed 5 times with wash buffer (PBS/0.05% Tween-20). A standard curve was generated from MMP-1 recombinant protein (R&D Systems) using the following concentrations of protein: 8ng, 4ng, 2ng, 1ng, 0ng. Samples and standards were run in triplicate. 100µL of either sample or standard was pipetted into the wells and the plate was covered in foil and incubated for one hour at 37˚C. The plate was washed five times with wash buffer. 100ng of biotinylated detection antibody (ACL2MMP1, Cedar Lane) was added to each well and the plate was incubated for 1 hour and 30 minutes at room temperature covered in foil. The detection antibody was removed by washing the plate five times with wash buffer. 100 µL of streptavidin/horseradish peroxidase (Sigma) was added to each well and the plate was incubated for 30 minutes at room temperature covered in foil. 100 µL of the tetramethylbenzadine (TMB, Sigma) was added to each well and the plate was incubated for 20-30 minutes, covered, depending on the intensity of the color change. The reaction was stopped with 100µL of 2.5N sulfuric acid. The plate was read in a plate reader at 450 nm within 30 minutes.

2.10 Immunoblotting

Ets-1 (N-276), cJun (H-79), cFos (H-125), cMet (C-12), and phospho-ERK (E-4) antibodies were purchased from Santa Cruz Biotechnology. Antibody for ETV1 was produced by PrimmBiotech using the peptide sequence published by the Jessell Laboratory (Columbia University). Nuclei were extracted[104] and quantified using the Coomassie Plus Protein Assay (Pierce). 10 μg of protein was loaded into a 4-12 % Bis Tris Gel and a Western Blot was performed using the antibodies described above.

2.11 Chromatin immunoprecipitation.

I. Cell plating, growth factor treatment and cell collection. T98 (1G/1G) and U251 (2G/2G) glioma cells were plated at a density of 2 x 10^6 cells per 100 mm plate in 10% DMEM. Three plates per condition (-HGF, +HGF, +HGF/+U0126) were plated to ensure the appropriate number of cells for the assay. Cells were allowed to attach to the plates overnight. 24 hours following plating, the cells are washed with warm 1x PBS and 10 mL serum free media is added to each plate overnight. The following morning the media is removed and 3 mLs of serum free media is added and cells are treated with 30 ng/mL HGF for 5 hours at 37˚C. Samples receiving U0126 (10μM) were treated for 30 minutes prior to the HGF treatment. Formaldehyde (37% stock, Fisher Scientific) was added at a final concentration of 1% directly to cell culture media for 8 minutes to crosslink the bound proteins to DNA. The media was removed and the cells were washed twice with ice cold PBS. Cells were scraped with a cell scraper, collected at the bottom of the plate in 4 mL ice cold PBS and added to a 15 mL conical tube. Like-conditions were pooled into the same conical tube and tubes were spun for 10 minutes at 1200 rpm to pellet the cells. PBS was removed from the cell pellet and the pellet was resuspended in Wash Buffer I (0.25% Triton X-100, 10

mM EDTA pH 8.0, 0.5 mM EGTA pH 7.5, 10 mM HEPES pH 7.5). The cells are spun for 10 minutes at 1200 rpm and wash buffer is removed. Cells were resuspended in Wash Buffer II (0.2 M NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 7.5, 10 mM HEPES pH 7.5) and spun for 10 minutes at 1200 rpm. The wash buffer was removed and the pellet was resuspended in 500 µL Lysis Buffer (150 mM NaCl, 25 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 1% Triton X-100, 0.1% SDS). Transfer samples to a pre-cooled 1.5 mL eppendorf tube. Cells were lysed by sonication, 20 fifteen second bursts, setting 2 on a Microson sonicator so that the average chromatin length was less than 1 kb. 1/5 of the 500 µL was pipetted into a pre-cooled 1.5 mL eppendorf tube to check the effectiveness of the sonication (see section II). The remaining sample was divided into three aliquot tubes (one for each of two antibodies and one for the No Antibody control). Aliquots were frozen in -80˚C until the sonication was determined to be satisfactory.

II. Analysis of sonicated DNA. Total volume of each sample is equilibrated to 200 µL with lysis buffer plus inhibitors. Samples were incubated in a 65˚C water bath overnight. The next morning, samples were cooled to room temperature and subjected to a phenol:cholorform extraction followed by DNA precipitation. Pellets were resuspended in 200 µL TE pH 8.0, 1 µL of 10 µg/mL RNase A was added and samples were incubated at 37˚C for 30 minutes to remove RNA from the sample. Following the RNA degradation, 1 µL of 20 mg/mL proteinase K was added to the sample in 1x proteinase K buffer (10 nM Tris pH 7.5, 5 mM EDTA pH 8.0, 0.25% SDS). Samples were incubated in a 42 ˚C water bath for one hour followed by a phenol:chloroform extraction and DNA precipitation. Pelleted DNA was resuspended in 60 µL TE ph 8.0, quantitated by spectrophotometry and 1 µg of DNA was run on a

0.8% agarose gel. The gel was examined to ensure that the average chromatin length was less than 1 kb. Once this was verified the aliquots were thawed on ice.

III. Immunoprecipitation, washes and elution. Sample volume was adjusted to 200 µL with lysis buffer that contained fresh inhibitors. The samples were precleared by adding 20 µL of protein A agarose beads (Roche) and rotated in 4°C for 1 hour. The beads were collected by centrifugation at 4000 rpm in 4°C for 5 minutes. The supernatant was pipetted into a fresh tube and 1 µg of antibody was added to each sample (5 µL of lysis buffer was added to the no antibody control tubes) and left to rotate overnight in 4°C. The next morning, 20 µL of protein A agarose beads were added to all the tubes and samples were rotated for 1 hour in 4°C. Tubes were spun at 4000 rpm for 5 minutes in 4°C and supernatant was discarded. The supernatant of the no antibody control tubes was transferred to a fresh tube and labeled total input. This sample was used to ensure that the input DNA contains the promoter of interest, in this case the MMP-1 promoter DNA.

IV. Washes, elution and cross-linking reversal. The beads were washed with 500 µL of RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% NP-40) by rotating at 4°C for 10 minutes. Beads were collected through centrifugation at 4000 rpm for 5 minutes in 4°C. The beads were then washed with high salt buffer (500 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 1% NP-40) by rotating at 4°C for 10 minutes. Beads were collected through centrifugation at 4000 rpm for 5 minutes at 4°C. The beads were then washed with LiCl wash buffer (250 mM LiCl, 50 mM Tris pH 8.0, 0.5% NaDoc, 1% NP-40) by rotating for 10 minutes at 4°C and the beads were collected through centrifugation at 4000 rpm for 5 minutes in 4°C. The protein A beads were then washed twice with TE pH 8.0 in the manner described

above and collected through centrifugation. Proteins bound to the beads were eluted by rotation in 200 µL of elution buffer (2% SDS, 10 mM DTT, 0.1 M $NaHCO_3$) for 15 minutes at room temperature. The beads were centrifuged at 4000 rpm for 5 minutes at room temperature and the fresh elution buffer was added. The supernatant from the two elutions was pooled together. To reverse the cross-linking, 0.2 M NaCl was added to the samples which were incubated in a 65˚C water bath for 4 hours. 1 mL of 100 % ethanol was added to the samples and then they were placed in a -20˚C freezer overnight to begin the precipitation. Phenol-chloroform extraction was performed on the total input samples were before the addition of the 100% ethanol step of the precipitation. The following morning the precipitation continued and DNA was resuspended in 180 µL TE pH 8.0. RNase A addition, proteinase K treatment and DNA precipitation was performed exactly as described in Section II above. DNA from the samples was resuspended in 20 µL TE pH 8.0 and DNA from the total input samples was resuspended in 60 µL TE pH 8.0

V. Sybr Green PCR. Total input samples were quantified with spectrophotometry as described above. The sample with the highest concentration of DNA was used to generate a standard curve for each primer set. The concentrations for the standards were 100ng, 20ng, 4ng, 0.8ng and 0ng. Standards were diluted so that the total amount of nanograms was contained within 1µL. Each standard was run in triplicate. A master mix of the PCR components was made in a total volume of 9µL. The master mix contained the following: 0.6 µM forward primer, 0.6µM reverse primer, 5µL Sybr Green reagent (Qiagen), H_2O to 9µL. The master mix was aliquoted into the wells of a 364 well plate and 1µL of either standard or sample was added to the well. The plate was covered in foil and carried, along with the plate cover, to the Nucleic

Acids Research Facility where it was run in the ABI 7700 Real Time PCR machine. Upon receipt of the results from the Nucleic Acids Research Facility, the data was analyzed. Any amplification in the No Antibody wells was considered background and subtracted from the samples. Samples were then averaged and plotted as nanograms of MMP-1 promoter DNA. MMP-1 distal promoter primer sequences were as follows: 5'-CAGTGGCAAGTGTTCTTTGG-3' and reverse 5'-CTCCCACCTTTCCCAC-TGTA-3'

Chapter 3

Association of a single nucleotide polymorphism in the matrix metalloproteinase-1 promoter with glioblastoma

Published in the International Journal of Cancer 2005 117:781-785

3.1 ABSTRACT

A key feature in the malignant behavior of glioblastoma is the tendency to invade host brain tissue surrounding the primary tumor site. Several members of the matrix metalloproteinase family are thought to contribute to this invasive capacity. A single nucleotide polymorphism has been described in the matrix metalloproteinase-1 (MMP-1) promoter that consists of either the presence or absence of a guanine nucleotide at position –1607. The presence of the guanine base creates a functional binding site for members of the ETS family of transcription factors and has been shown to increase MMP-1 transcription. The purpose of this study was to characterize this polymorphism in human glioblastoma. Promoter genotyping was performed on brain tumor tissue obtained from 81 patients and compared to 57 healthy individuals. The 2G/2G genotype is more prevalent in glioblastoma tissue compared to healthy individuals ($p=0.01$). mRNA and protein expression were measured in a subset of brain tumor and normal brain tissue samples. MMP-1 protein levels are significantly higher in glioblastoma tissue compared to normal brain ($p=0.001$). Electromobility shift assays and promoter assays were performed to assess binding capability and transcriptional activity respectively. Proteins present in glioma cell lines can specifically bind the 2G promoter probe. MMP-1 transcription is significantly higher in cells transfected with the 2G promoter when compared to cells transfected with the 1G promoter ($p<0.02$). This polymorphism may provide a mechanism for increased expression of MMP-1 in malignant gliomas via elevation of MMP-1 mRNA transcription and may underlie the invasive phenotype.

3.2 INTRODUCTION

Gliomas account for 44% of all primary central nervous system tumors and of these the most common is the highly malignant glioblastoma multiforme (GBM)[5]. Despite significant improvements in the diagnosis and treatment for patients with a GBM, this primary brain tumor remains essentially incurable. Although surgery and radiotherapy substantially improve patient survival[2], 95% of patients have a mean survival of two years following diagnosis. Patients foregoing treatment have a mean survival time of four months. The characteristic ability of GBMs to invade surrounding normal tissue makes total surgical resection virtually impossible.

Matrix metalloproteinase-1 (MMP-1), also known as collagenase-1, is a member of the MMP family of zinc dependent endopeptidases, which have the ability to cleave substrates in the extracellular matrix. Substrates for MMP-1 include collagen types I, II, III, VII, and X, gelatin, entactin, aggrecan, and tenascin. Until recently it was thought that MMPs were responsible mainly for the degradation of extracellular matrix components. However it has become clear that the MMP family has a wide range of other influences on biologic processes including the generation of bioactive proteins[19,105,106] . Examples include the cleavage of the proteoglycan perlecan to release fibroblast growth factor (FGF), cleavage of insulin like growth factor binding protein (IGF-BP) to release IGF, and cleavage of pro-tumor necrosis factor α (pro-TNFα) to release TNFα[105].

MMP-1 has been less well characterized in human brain tumors than other MMPs such as MMP-2 and MMP-9[107-112]. This may be due preconceptions that a collagenase would not be expected to play a prominent role in human brain tumors due to the absence of significant quantities of collagen I in the brain. Nevertheless, MMP-1

has in fact been shown to be present in these tumors. MMP-1 protein levels increase with increasing tumor grade and correlate with increased glioma invasiveness[45,46,113-115]. In the absence of significant quantities of collagen in normal brain or brain tumor tissue, recent attention has focused on the ability of MMPs to act enzymatically on other ECM components and pro-forms of specific cytokines.

A functional single nucleotide polymorphism (SNP) is known to occur at position -1607 in the MMP-1 promoter[47]. It consists of either the presence or absence of a guanine nucleotide adjacent to a pre-existing guanine nucleotide at -1606. These two allelic phenotypes are referred to as the 2G or 1G allele, respectively. The presence of the guanine nucleotide (2G) creates a core DNA binding site for members of the ETS family of transcription factors and has been shown in other cell types to lead to a significant increase in MMP-1 transcriptional activity[47]. In several types of cancer studied, the incidence of the 2G allele is significantly higher in aggressive and metastatic tumors[55-57]. Due to the strong implication of MMP-mediated proteolysis in glioma progression, we sought to determine the prevalence of the 2G allele in GBM patients and the effects this polymorphism elicits on MMP-1 transcription, mRNA and protein levels.

3.3 MATERIALS AND METHODS

3.3.1 Cell Lines. Human glioma derived cell lines (U87 and T98) were obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin in a humidified incubator at 37° C with 5% CO_2. LN-Z308 (null p53) human glioma derived cells, a kind gift from Dr. Erwin VanMeir, were maintained in the same conditions.

3.3.2 Primary Human Samples. Tumor samples were obtained from 81 patients who underwent surgery for glioblastoma. Tumor tissue obtained at time of craniotomy was dissected free of adventitial material, snap-frozen in liquid nitrogen, and maintained in a tissue bank at –80°C in accordance with an approved protocol from our Institutional Review Board. Upon confirmation of diagnosis of glioblastoma multiforme by a neuropathologist, genomic DNA was extracted using the QiaAmp DNA Mini Kit (Qiagen, CA) and quantified by spectrophotometry. DNA was extracted from buccal swabs of 57 healthy volunteers also in accordance with an approved protocol from our institution, using the QiaAmp DNA Mini Kit and quantified by spectrophotometry.

3.3.3 Genotyping. Analysis of the SNP in the MMP-1 promoter was conducted on DNA from 81 GBM patients and DNA from 57 healthy volunteers following the protocol previously described by Zhu *et al.*[56]. In this protocol, genomic DNA is amplified for a 269 base pair product using the polymerase chain reaction (PCR) with a pair of primers directed at the MMP-1 promoter sequence. The reverse primer is modified to incorporate an *Alu I* recognition site (AGCT) at the 1G allele. That is, the 1G allele retains this sequence, whereas the presence of the additional guanine nucleotide in the 2G allele disrupts this recognition sequence. PCR primer sequences were as follows:

forward 5'-TGACTTTTAAAACATAGTCTATGTTCA-3'; and reverse 5'-TCTTGGATTGATTGAGATAAGTCATAGC-3'. The PCR product was digested overnight at 37°C with one unit of *Alu I* (New England Biolabs, MA) 1x reaction buffer and sterile water to a total volume of 30 µL. Digestion products were separated on a 4% agarose gel for 1 hour at 100 V and visualized with ethidium bromide. The 1G homozygous individuals (1G/1G) were represented by bands at 241 and 28 bp, 2G homozygous individuals (2G/2G) were represented by a single band at 269 bp while the heterozygous individuals (1G/2G) were represented by bands at 269, 241 and 28 bp. An example of the PCR products from each of these genotypes is demonstrated in Figure 8A.

3.3.4 Sequencing. The genotype of the gliomas and normal tissue was confirmed by selecting random samples and subjecting them to PCR previously described by Hirata *et al.*[48], followed by direct DNA sequencing performed at the University of Iowa Sequencing Facility. PCR primer sequences were as follows: forward 5'-CAGTGGCAAGTGTTCTTTGG-3' and reverse 5'-CTCCCACCTTTCCCACTGTA-3'.

3.3.5 Nuclear Extraction and Electrophoretic Mobility Shift Assay. Nuclear extracts from U87, T98 and LN-Z308 cells were obtained as described previously[104] and dialyzed for one hour on 0.025µ filter (Millipore) in Dialysis Buffer (20mM HEPES, 20% glycerol, 100mM KCl, 0.2mM EDTA, 0.2mM PMSF, and 0.5 mM DTT). 10µg of nuclear extract was incubated for 20 minutes at room temperature with 9pmol of ^{32}P end-labeled double stranded oligonucleotide (5'-TAGAAAGGATATGAC-TTATCTCA-3') in 20µL binding buffer (1µg salmon sperm, 5% Ficoll, 5mM $MgCl_2$). For competition assays 90pmol (10 fold excess) of unlabeled double stranded oligonucleotide encompassing either the Ets-1 binding site (5'-GGAGG-

AGGGCTGCTTGAGGAAGTATAAGAAT-3') or the NFkB binding site (5'-AGTTGAG-GGGACTTTCCCAGGC-3') was added to the binding buffer and labeled 2G probe. DNA-protein complexes were separated by electrophoresis at 4°C through a 6% native polyacrylamide gel for 2 hours at 210 volts, dried for one hour at 80°C and exposed to autoradiography film overnight.

3.3.6 Transfection and Luciferase Assays. Cells were transiently transfected in triplicate using the LipofectAMINE 2000 reagent (Invitrogen, CA) using 2 µg of MMP-1 promoter/reporter plasmids (a kind gift from Dr. Constance Brinckerhoff) and 4 µL LipofectAMINE 2000. Lysates were collected after 24 hours and luciferase activity was measured in relative light units (RLUs) on the FLUOstar 403 (BMG Lab Technologies, Germany). Samples were normalized to the internal control plasmid Renilla which was transfected into the cells at the same time as the MMP-1 promoter.

3.3.7 RNA Extraction and Real Time PCR. Total RNA was extracted from 6 normal brain samples and 22 glioma tissue samples of known genotype (5 1G/1G, 9 1G/2G, and 8 2G/2G) by following the TRIzol protocol supplied by the manufacturer (Invitrogen, CA). RNA was quantified using spectrophotometry and sent to the Molecular Core of the Virginia Commonwealth University-Massey Cancer Center Nucleic Acids Research Facilities for Real Time PCR analysis of MMP-1 mRNA levels using the ABI 7900 Sequence Detection System.

3.3.8 Protein Extraction and ELISA. Total protein was extracted from 6 normal brain samples and 26 glioma samples of known genotype (6 1G/1G, 10 1G/2G, and 10 2G/2G) using Tissue Protein Extraction Reagent (T-PER, Pierce, IL) and quantified using the Coomassie Plus Protein Assay Reagent Kit (Pierce, IL). 10 µg of total

protein was subjected to a sandwich enzyme immunoassay. Capture and detection antibodies were obtained from Cedar Lane (Canada).

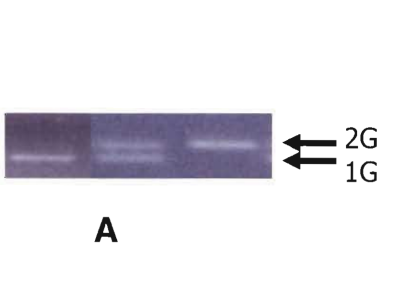
3.3.9 Statistical Analyses. Differences in the allele frequencies between normal and GBM patients were studied by contingency table analysis using the Chi-square test (two tailed). A null hypothesis probability of less than 0.05 was considered to be significant. For comparisons of MMP-1 promoter activity (Luciferase assays), normalized relative light unit (RLU) values were obtained from triplicate samples, and compared using student's unpaired t test. Odds ratio was calculated using JMPin® 4.0.4 (SAS Institute, Inc.) software.

3.4 RESULTS

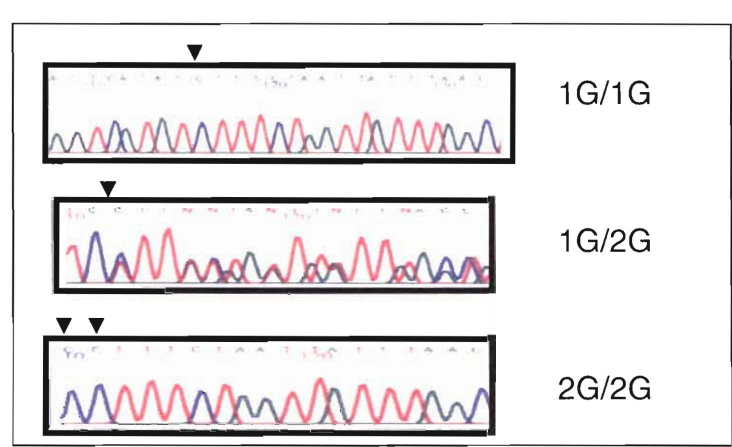
We determined the MMP-1 promoter genotype in 81 samples from patients with GBM and in 57 samples of buccal swabs from healthy volunteers by PCR and subsequent restriction enzyme digest (Figure 8a). DNA from random samples was sequenced to verify PCR results (Figure 8b). The allelic variation of the MMP-1 promoter was significantly different when comparing DNA from healthy volunteers to that of the GBM samples (Table 4 X^2=6.5 p= 0.03). The allelotype frequency in a demographically similar normal population, published by Ye *et al.*[53], was also significantly different from that of the GBM population tested here (Table 5, p=0.001). Comparison of pooled data for the normal distribution published by multiple investigators[53,54,56,116] to the allelic distribution of the GBM samples generated in this study also reveals a significant difference (Table 5, p=0.01). Additionally, the 2G/2G genotype was more prevalent in the GBM samples (42%) compared to data from our control DNA (21%, p=0.018).

To determine if proteins present in nuclear extracts of human glioma cell lines were capable of binding the ETS DNA consensus sequence found at –1607 in the MMP-1 promoter, we conducted electromobility shift assays with a probe designed to mimic the 2G promoter (Figure 9). Two DNA-protein complexes are present when nuclear extracts are incubated with the 2G probe (*Lanes 2, 7, and 12*). This complex can be competed with the addition of ten-fold excess unlabeled 2G probe (*Lanes 3, 8, and 13*). Addition of ten-fold excess unlabeled probe containing the Ets-1 binding site (*Lanes 4, 9, and 14*) also results in a decrease in binding to the labeled 2G probe suggesting that the Ets-1 protein is binding to the 2G probe. Ten-fold excess unlabeled probe containing the binding site for NFkB (*Lanes 5,10 and 15*), a protein not

Figure 8. Genotyping of the MMP-1 promoter polymorphism in healthy individuals and individuals with glioblastoma. A. Representative agarose gel of the PCR amplification of genomic DNA sequences. 1G/1G individuals are represented by a 241bp fragment. A single fragment of 269-bp represents the 2G/2G and the 1G/2G heterozygous individuals are represented by two DNA fragments of 269 and 241bp. B. Representative sequence of the three genotypes using reverse primer.



A



B

Table 4. Relationship between MMP-1 Promoter Polymorphism and Glioblastoma

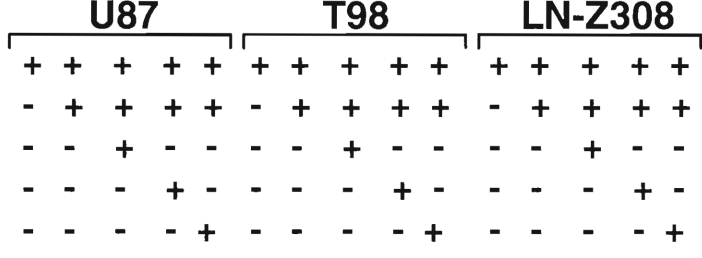
Genotype	Cases n	%	Controls n	%	p-value	Odds Ratio	95% CI
1G/1G	13	16%	15	26%		0.31	0.11-0.81
1G/2G	34	42%	30	53%		0.40	0.17-0.89
2G/2G	34	42%	12	21%	0.03*	reference	

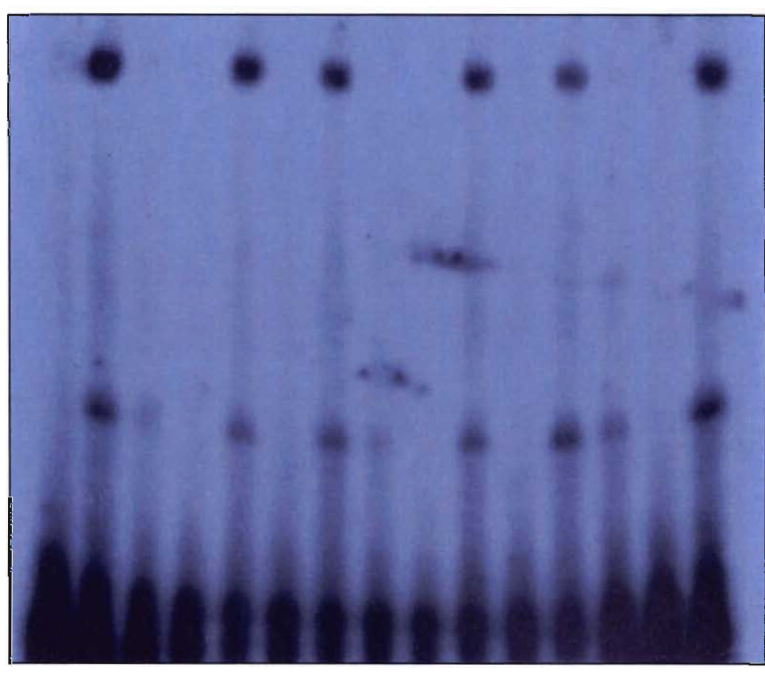
*p-value indicates difference in distribution of MMP-1 genotype between normal and GBM patient populations

Table 5. Comparison of allele frequency between DNA from high grade glioma samples generated in this study with DNA from healthy volunteers from previously published studies.

	MMP-1 Promoter Genotype, n(%)			p-value relative to GBM data	Data Source
	1G/1G	1G/2G	2G/2G		
GBM DNA (n=81)	13 (16%)	34 (42%)	34 (42%)		
Normal DNA (n=57)	15 (26%)	30 (53%)	12 (21%)	p=0.03	This study
Normal DNA (n=142)	41 (29%)	68 (48%)	33 (23%)	p=0.001	(22)
Normal DNA (n=1097)	280 (26%)	518 (47%)	299 (27%)	p=0.01	(20, 22-24)

Figure 9. Binding interactions of the polymorphism in the MMP-1 promoter. Electromobility shift assays were performed with three human glioma cell lines. ^{32}P labeled probe corresponds to 23 bp in the promoter flanking the polymorphism site at position -1607.


U87
T98
LN-Z308
Labeled MMP-1 probe + + + + + + + + + + + + + + +
10 ug nuclear extract - + + + + - + + + + - + + + +
10x cold MMP-1 probe - - + - - - - + - - - - + - -
10x cold Ets-1 probe - - - + - - - - + - - - - + -
10x cold NFkB probe - - - - + - - - - + - - - - +


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

capable of binding to the 2G probe, does not reduce the binding of proteins to the 2G probe. This indicates that the binding of proteins to the 2G probe is specific. We performed supershift EMSAs with antibodies for Ets-1 and Ets-2 but were unable to detect a shift in the binding pattern (data not shown). We next wanted to assess the influence of the additional ETS binding site in the MMP-1 promoter on transcriptional activity in human glioma cells. We performed promoter reporter assays using luciferase reporter constructs obtained from Dr. Brinckerhoff[47]. These constructs are driven by the 4.3 kb MMP-1 promoter and differ at position –1607 with respect to the presence (2G construct) or absence (1G construct) of a guanine nucleotide. We measured the transcriptional activity of the MMP-1 promoter following transient transfection of the 1G or 2G construct into three human glioma cell lines. We observed approximately a three fold increase in MMP-1 transcriptional activation in all cell lines tested ($p<0.02$, Figure 10.)

Next we examined a subset of tissue, both GBM and normal brain, of known genotype, to determine if the presence of the 2G/2G genotype correlates with increased MMP-1 mRNA and protein expression. Total MMP-1 protein levels are significantly higher in the glioma tissue examined compared to normal brain (Figure 11B, $p=0.001$). The elevated mRNA and protein levels were not significantly different among GBM samples with the exception that the decrease in protein expression of the 2G/2G samples compared to that from the 1G/2G samples was statistically different ($p=0.02$, Figure 11).

Figure 10. Transcriptional activation of the MMP-1 promoter. MMP-1 promoter constructs containing 1G or 2G at position –1607 were transiently co-transfected with internal control gene, Renilla into three human glioma cell lines. Activity was measured by luminescence. Asterisk indicates a p-value of 0.02. Data are presented as standard errors of the mean of six independent experiments.

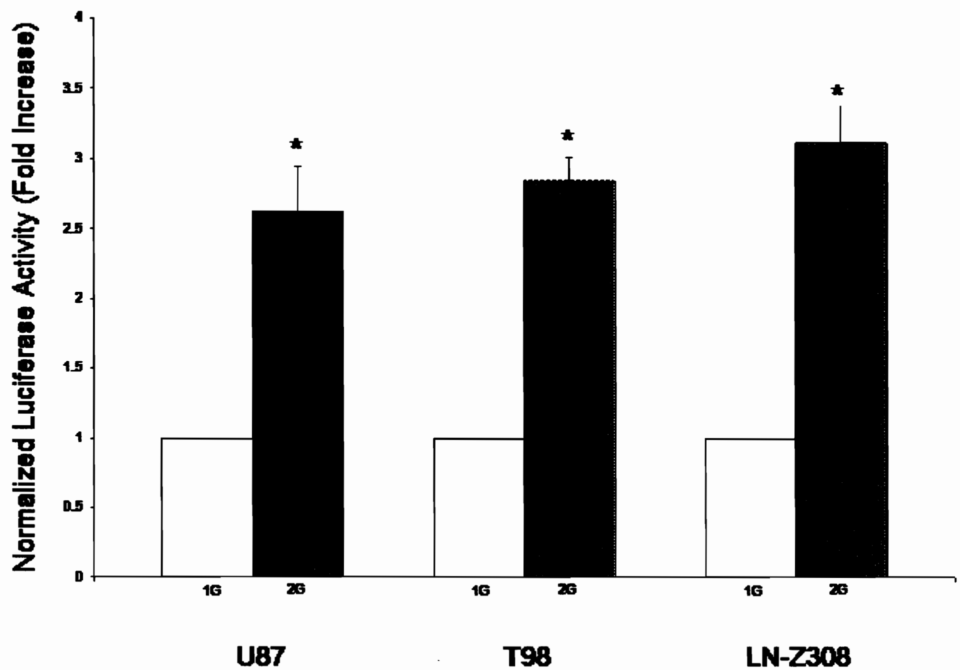
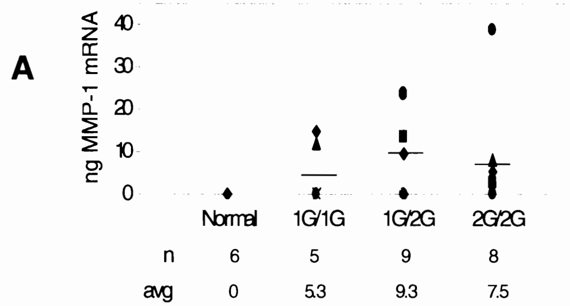
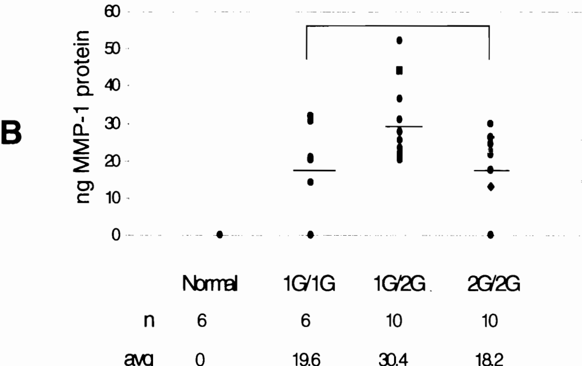

Normalized Luciferase Activity (Fold Increase)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
1G
2G
1G
2G
1G
2G
U87
T98
LN-Z308

Figure 11. MMP-1 mRNA and protein levels in normal brain and GBM samples of differing genotype. mRNA and protein was isolated from normal brain and GBM samples and subjected to Real Time PCR and ELISA to determine expression levels of MMP-1.

A



B



3.5 DISCUSSION

MMP-1 has been implicated in tumor invasion, metastasis, and angiogenesis[21]. The presence of the additional guanine nucleotide at position -1607 in the MMP-1 promoter was discovered in a highly invasive melanoma cell line and since has been studied in several types of cancer. Cancers that have been examined for the MMP-1 promoter genotype include melanoma[53], lung[56], renal cell carcinoma[48], cervical carcinoma[59], oral squamous carcinoma[52], breast[58,116], ovarian[54,55], endometrial carcinoma[49], and colorectal carcinoma[57]. In all but two studies (breast cancer[58] and melanoma), there was a significant difference in the distribution of the genotypes between the control population and the tumor population. Despite the lack of significance in genotype distribution in breast and melanoma, both of these cancers show functional differences in tumors containing a 2G allele when compared to the tumors containing the 1G allele. Breast cancer patients with a 2G allele had a higher rate of metastasis than the patients with the 1G allele as indicated by tumor positive lymph nodes. Similarly, the cutaneous malignant melanomas containing the 2G allele were more invasive than those with the 1G allele. A correlation between the 2G allele and metastases is also present in colorectal carcinoma. It has also been reported that the 2G allele has been implicated in significantly decreasing the age of onset of lung cancer in male smokers.

The experiments described in this study investigated the distribution of the MMP-1 promoter polymorphism in tissue samples from patients with glioblastoma multiforme and compared this to the distribution in the normal healthy population. Although the 2G/2G genotype has been reported to be significantly higher in other

types of cancer, this is the first report of a significant difference in the MMP-1 promoter genotype in human glioblastomas.

The results from the gel shift assays indicate that there are proteins present in glioma cell lines capable of binding to the 2G promoter. The competition assay with the unlabeled Ets-1 probe suggests that Ets-1 is a protein that is capable of binding to this ETS site. The inability of the Ets-1 antibody to elicit a supershift does not eliminate the possibility that this protein is binding the probe. The limitations of using antibodies for this type of binding experiment are well known. In fact, in the seminal paper describing this polymorphism, Rutter *et al*[47] also failed to detect a supershift with antibodies for various ETS family members. We are currently conducting more specific binding assays to address the interactions between ETS proteins and the 2G promoter. We also examined the effect of the presence or absence of the guanine nucleotide on the transcriptional activity of the MMP-1 promoter in three glioma cell lines. These experiments demonstrated that the 2G promoter construct results in a significant increase in transcriptional activity over the 1G promoter construct. Taken together, the results from the EMSAs and the transcriptional activity assays, strongly suggest that the addition of the guanine nucleotide in the MMP-1 promoter creates a functional binding site that is relevant to glioma biology.

This study also demonstrates that MMP-1 protein levels are significantly higher in GBMs when compared to normal brain. This data is in agreement with studies conducted by Nakagawa *et al.*[46] and Nakano *et al.*[45] Comparison of total MMP-1 levels in a subset of GBM samples of varying genotypes reveals that the tumors with the 1G/2G genotype express the highest levels. Further studies are needed to determine if there is a difference in the ratio of active versus the pro-form of MMP-1. Additionally,

due to the high heterogeneity of the tumors it would be interesting to examine MMP-1 activity levels within the invasive population of tumor cells.

The transcriptional regulation of MMP-1 is highly complex and involves multiple transcription repressors, co-activators and enhancers. In light of this complexity, the relationship between the genotype and MMP-1 expression may not be surprising. For example, the p53 status of the tumor could influence the levels of MMP-1 as it has been shown that wild-type p53 suppresses MMP-1 transcription[67-69]. Brinckerhoff *et al.*[18] propose a model that addresses the likelihood of the 2G allele contributing to pathologic processes. The model states that several molecular events must take place in addition to the presence of the 2G allele. Examples of these events include the overexpression of transcription factors binding the 2G promoter, driven by unregulated autocrine or paracrine signaling and the deregulation of transcription repressor function.

One interpretation of this data is that the presence of the 2G SNP is one important mechanism, of several potential pathways, by which transformed glial cells have an increased invasive behavior. This would support the concept that increased MMP-1 expression is an important contributor to the invasive behavior which is a *sine qua non* in glioblastoma, and that individuals with the 2G SNP have an inherent tendency toward this invasive phenotype by virtue of the increased activity of the 2G promoter. Extension of this reason would hypothesize that individuals without the 2G SNP who develop glioblastoma have acquired other molecular genetic events that result in increased MMP-1 expression and glioma cell invasiveness. Further characterization of the mechanisms by which MMP-1 expression is controlled in glial cells will hopefully shed further light on this question.

Chapter 4

Increase in MMP-1 2G Promoter Transcription from Hepatocyte Growth Factor Stimulation is caused by AP1 and Ets-1 cooperativity in Gliomas

4.1 ABSTRACT

Gliomas remain one of the deadliest tumors despite improvements in treatment approaches and technology. The invasive nature of the tumor cells renders them incurable with a mean survival rate of 9-12 months following diagnosis. Enzymes responsible for this invasive nature include the matrix metalloproteinase family. MMP-1 is a member of this family which has been well studied in many types of invasive tumors, with gliomas being an exception. A polymorphism in the promoter region leads to increases in transcription from the MMP-1 promoter in gliomas. It consists of the presence (2G) or absence (1G) of a guanine nucleotide at position -1607. The additional guanine nucleotide creates a binding site for ETS transcription factors and combined with the AP-1 binding site at position -1602 creates a Ras Responsive Element in the distal promoter. We performed Western blots to establish that HGF/SF stimulated the MAP kinase pathway in glioma cells and that this lead to increases in AP-1 proteins. Promoter assays indicate that HGF/SF treatment results in increases in MMP-1 transcriptional activity from the 2G promoter. This activity is significantly higher than the untreated 2G promoter and higher than both the treated and untreated 1G promoter. Chromatin immunoprecipitation assays demonstrate that the transcription factors bound to the 2G promoter are different from those bound to the 1G promoter and that Ets-1 and AP-1 are bound to the additional Ras Responsive Element in the 2G promoter. Our data indicate that this is one potential mechanism responsible for the increases in MMP-1 transcription in glioma cells containing a 2G MMP-1 allele.

4.2 INTRODUCTION

Although malignant brain tumors account for only 2% of all adult cancers, the poor survival rate associated with these tumors results in a disproportionate number of years of life lost compared to other cancers. Patients with glioblastoma multiforme (GBM), the most malignant glioma, have a median survival rate of 9-12 months following diagnosis. Even with impressive advances in both neurosurgical techniques and radiation therapy over the past three decades, GBMs recur in over 80% of patients even if the lesion is in an area with a wide surgical margin. A major feature that underlies the malignant behavior of GBMs is the ability of glioma cells to aggressively infiltrate surrounding brain tissue. Included in the factors that play a role in the invasive nature of glioma cells are certain members of the matrix metalloproteinase (MMP) family. We have previously reported that MMP-1 is present in high grade gliomas and that the single nucleotide polymorphism (SNP) discovered in melanomas[47] is present in human gliomas[44].

The SNP in the MMP-1 promoter which consists of the presence or absence of a guanine nucleotide at position -1607 was first discovered in a highly aggressive melanoma cell line and has since been studied in many types of invasive tumors[47,48,53,57]. The addition of the guanine base creates a consensus binding site for members of the ETS family of transcription factors (Figure 12a). The allele with the additional guanine base is designated the 2G allele and the allele without the additional guanine base is designated the 1G allele. The 2G allele has increased levels of transcription when compared with the 1G allele in gliomas[44], breast cancer cells[117] and melanomas[47]. MMP-1 transcription is known to be regulated by growth factors and cytokines. One such growth factor known to induce MMP-1 transcription is hepatocyte

growth factor (HGF). HGF, originally identified as a potent mitogen for primary rat hepatocytes, is overexpressed in 80% of gliomas. It is identical to scatter factor (SF) which increases cell motility and invasiveness[93] HGF/SF binds to the tyrosine kinase receptor cMet which is overexpressed in 100% of gliomas[88]. HGF/SF is expressed in both normal human astrocytes and gliomas however GBMs contain a higher level than both lower grade gliomas and normal brain. Expression of HGF/SF and cMet is correlated with a decrease in patient survival, poor prognosis, enhanced tumorigenicity, and increased cell invasion[98,102,118,119]. Inhibition of endogenous HGF/SF and cMet expression in gliomas can reverse their malignant phenotype. Knockdown of HGF/SF inhibits the growth of GBM xenografts, promotes apoptosis, inhibits proliferation and angiogenesis and promotes animal survival[102].

Binding of HGF/SF to its receptor leads to, among other events, increases in MMP-1 promoter activity[94-97,119]. One mechanism responsible for this increase in MMP-1 is the activation of the Ets-1 transcription factor. Ets-1 is the founding member of the ETS family of transcription factors. ETS proteins bind to a conserved winged helix-turn-helix binding domain containing the sequence GGAA/T[120]. These proteins do not associate as dimers but form complexes with transcription factors in unrelated families. Without a binding partner they have only weak trans-activating abilities[77].

Jun and Fos are well characterized binding partners of ETS proteins. Jun and Fos proteins bind as dimers to AP-1 regulatory elements in the promoter and enhancer regions of many genes. They are members of the bZIP family of transcription factors which contains a highly conserved basic region involved in DNA binding. They also contain a heptad repeat of leucine residues, known as the leucine zipper, which is required for dimerization. Dimerization is necessary for DNA binding[121]. Jun and Fos

proteins can form multi-protein complexes with transcription factors in unrelated families that form at regulatory elements in mammalian promoters. These multi-protein complexes are more stable than complexes formed by individual transcription factors and often form in response to signal transduction pathways[122].

An example of an element that multi-protein complexes bind to is the Ras Responsive Element (RRE). It contains an ETS binding site adjacent to an AP-1 site. ETS and AP-1 bind to the RRE in promoters of genes involved in migration and invasion, notably the MMP promoters. MMP-1 contains an RRE in the proximal promoter (-87 to -77) and depending on the genotype of the cell, an additional RRE in the distal promoter (-1607 to -1602). In order to enhance binding to the RRE, Ras dependent activation of both Ets-1 and AP-1 proteins is necessary. Binding of HGF/SF to cMet is sufficient to activate the GTPase Ras. Activation of Ras in turn activates the Raf kinase. Raf phophorylates MEK kinase which phosphorylates ERK kinase (Figure 3c). ERK kinase docks onto the pointed domain of Ets-1 and phosphorylates threonine 38[120].

The addition of the guanine nucleotide creates a functional binding site for members of the ETS family of transcription factors. This binding site is adjacent to an AP-1 binding site and therefore forms an additional Ras Responsive Element in the distal 2G promoter. This element is responsible for synergistic increases in transcription when stimulated by Ras. To date no other group has investigated the mechanism responsible for increased transcription from the 2G promoter in gliomas. We evaluated the responsiveness of the MMP-1 distal promoter to HGF/SF stimulation in gliomas. The results from our study indicate that HGF/SF treatment induces binding of cJun, cFos and Ets-1 to the additional RRE in the MMP-1 2G distal promoter. This

binding is one possible mechanism for the increase in transcription from the 2G promoter in gliomas.

4.3 RESULTS

T98 and U251 glioma cells have different MMP-1 promoter status. We genotyped a panel of glioma cell lines using PCR followed by restriction digest and chose two cell lines that differed in their promoter status (Figure 12b). T98 was homozygous for the 1G promoter and U251 was homozygous for the 2G promoter. Samples were also sequenced to verify the MMP-1 promoter genotype (Figure 12c). The sequencing confirmed that the T98 cell line is 1G/1G and that the U251 cell line is 2G/2G. To verify that these two glioma cell lines contained cMet, the HGF/SF receptor, we extracted protein from T98 and U251 and conducted Western blots for cMet (Figure 13a). This confirmed that both cell lines contained the receptor for HGF/SF.

HGF/SF induces MMP-1 mRNA in glioma cells and may be genotype dependent. Although it is known that the addition of HGF/SF leads to an increase in both MMP-1 mRNA and protein levels in other cell types, the link between HGF/SF and induction of MMP-1 in gliomas has not been examined. To determine if HGF/SF affects MMP-1 mRNA levels in gliomas and if this effect is based on the promoter status, we performed real time PCR on T98 and U251 mRNA (Figure 13b). The T98 cells, which contain a 1G/1G promoter, had a higher basal level of MMP-1 expression compared to the 2G/2G cells, U251. HGF/SF stimulation increased the mRNA levels in T98 cells approximately two-fold. In contrast, MMP-1 levels were increased over ten fold in response to the HGF/SF treatment.

HGF/SF activates the MAP kinase pathway in gliomas cells. We next wanted to determine if the increase in MMP-1 mRNA levels was due to activation of the MAP kinase pathway. We used U251 cells to analyze ERK activity because they had a

Figure 12. Glioma cell lines differ in their MMP-1 promoter status. a. Schematic of the MMP-1 promoter. The proximal promoter of the 1G and 2G alleles are identical in that both alleles contain ETS and AP-1 binding sites. The single nucleotide polymorphism in the distal promoter distinguishes the 1G allele from the 2G allele. The 1G allele contains a consensus binding site for the AP-1 family of transcription factors whereas the 2G allele contains sites for both the ETS and AP-1 binding proteins. b. Genotyping of T98 and U251 cell lines. Genomic DNA was extracted from T98 and U251 glioma cells. DNA is subjected to PCR, digested with Alu I overnight and run on a 4% agarose gel. 1G/1G samples retain the digest sequence while the 2G/2G cells do not, therefore 1G/1G DNA migrates further through the gel. c. DNA sequencing of T98 and U251 cell lines. Genomic DNA extracted from T98 and U251 glioma cells was sent to the University of Iowa Sequencing Facility to verify the genotype.

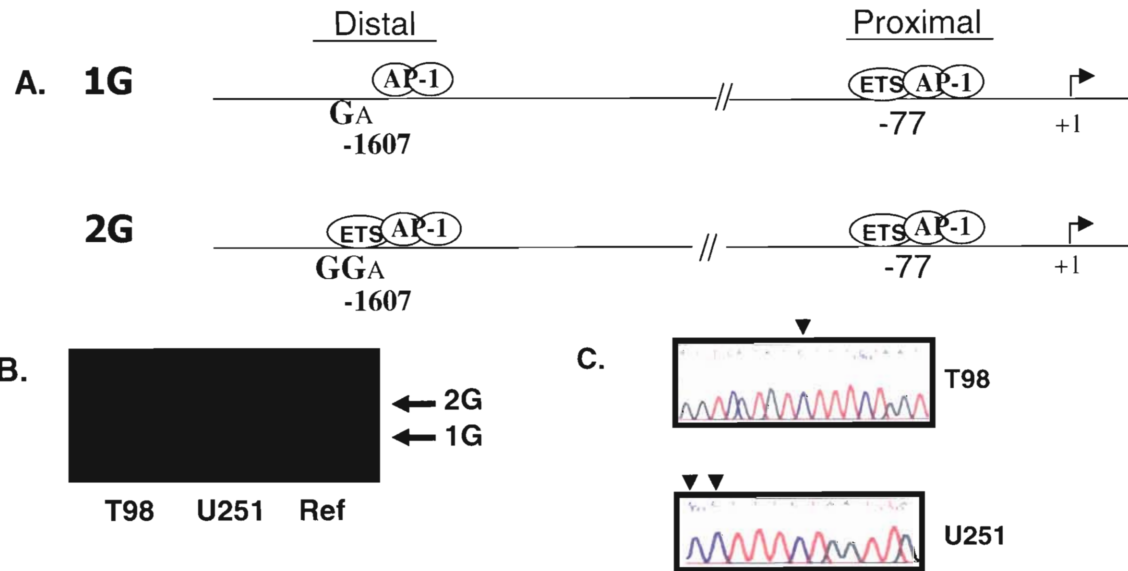
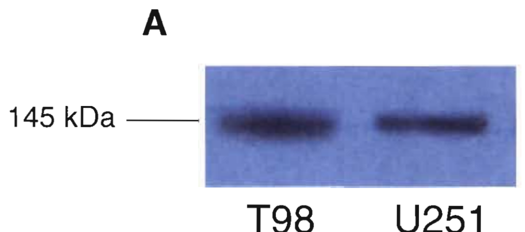
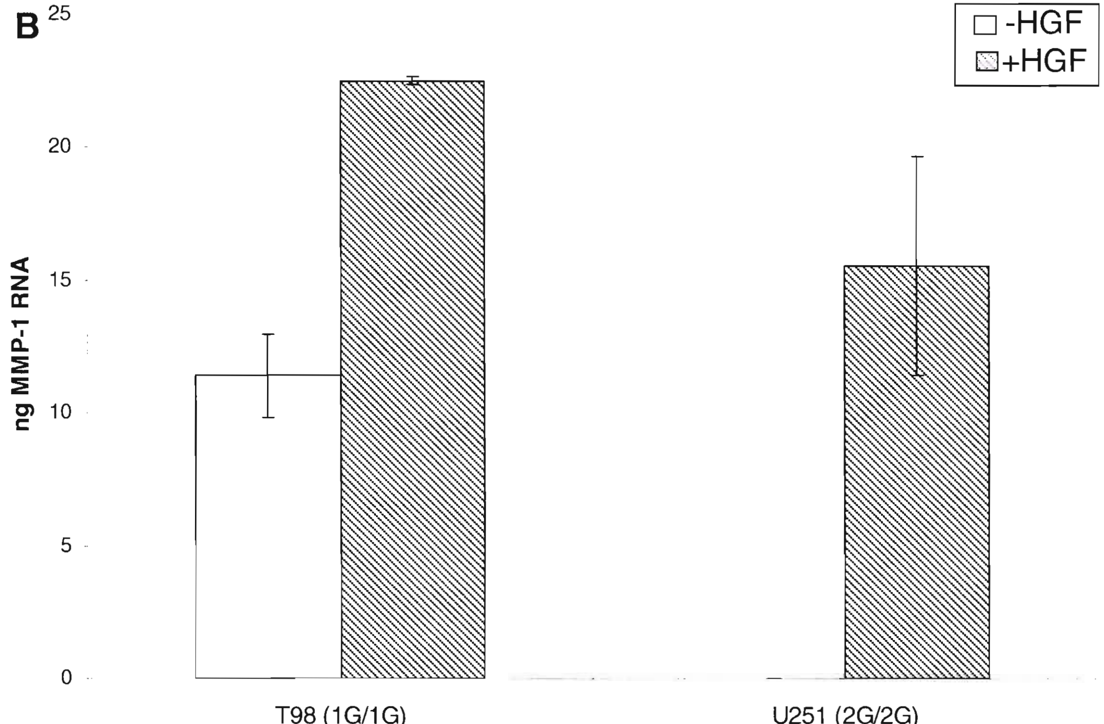

Distal
Proximal
A. 1G
AP-1
ETS AP-1
GA
-1607
-77
+1
2G
ETS AP-1
ETS AP-1
GGA
-1607
-77
+1
B.
2G
1G
T98 U251 Ref
C.
T98
U251

Figure 13. MMP-1 genotype determines responsiveness to HGF/SF treatment in glioma cell lines. A. Western blot of T98 and U251 glioma cells probed with the cMet antibody (C-12, Santa Cruz). b. T98 and U251 glioma cells were treated with 30 ng/mL HGF for 12 hours. Total RNA was extracted, quantified and sent to the Nucleic Acids Research Facility at VCU for Real Time PCR analysis of MMP-1. Data is represented as a ratio of MMP-1 RNA to the internal control 18S RNA.

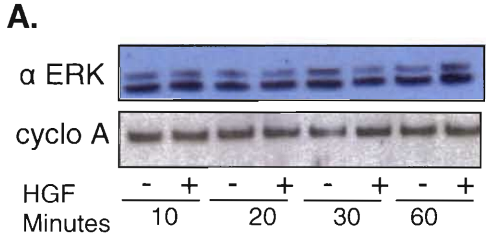

A
145 kDa
T98
U251

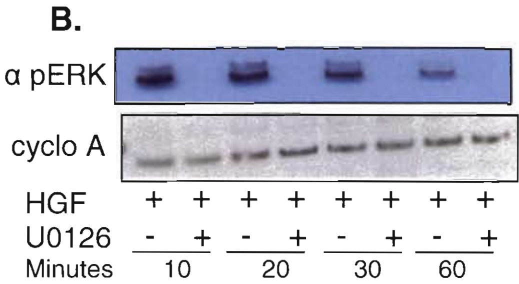

B
-HGF
+HGF
ng MMP-1 RNA
0
5
10
15
20
25
T98 (1G/1G)
U251 (2G/2G)

more robust response to HGF/SF than the T98 cells. We stimulated the cells with HGF/SF for the indicated time and performed Western blot analysis on the nuclear proteins. We used antibodies for either total ERK or the active form, phosphoERK, to probe the Western blots (Figure 14). Total ERK levels did not change in response to the HGF/SF treatment however phosphoERK appears in the nucleus within 10 minutes of stimulation. Addition of the MEK inhibitor, U0126, prevented the activation of phosphoERK. This indicates that phosphorylation of ERK is dependent on MEK activation.

HGF/SF stimulated MMP-1 transcription is higher in 2G/2G gliomas cells compared with 1G/1G cells. Using transient transfection assays, we have previously determined that the basal activity of the 2G promoter is approximately three fold higher than the basal activity of the 1G promoter in three gliomas cell lines (U87, T98 and LN-Z308)[44]. To determine if the addition of the ETS binding site in the distal MMP-1 2G promoter influences the response to HGF/SF we performed luciferase reporter assays with both the 1G and 2G full length promoters (Figure 15a). Both the 1G and the 2G MMP-1 promoter constructs (a kind gift from Dr. Brinckerhoff) respond to the addition of HGF/SF. The increase in activity between the 1G and 2G promoters is similar at three hours. At 5 hours the 2G promoter has a much more robust response than the 1G promoter and this increase in response relative to the 1G promoter continues for 24 hours. The addition of a MEK inhibitor, U0126, inhibits this response in both the 1G and 2G promoters (Figure 15b). There is no difference in inhibition between the 1G and 2G promoters until the 9 hour timepoint. This inhibition continues until the 24 hour timepoint.

Figure 14. The ERK kinase is activated in U251 glioma cells after HGF treatment. U251 glioma cells were treated with 30 ng/mL HGF for the indicated timepoint. Cells receiving the inhibitor were treated with 10 uM U0126 thirty minutes prior to HGF treatment. Nuclear proteins were extracted and 10 ug was used for a Western blot. a. Total ERK (Cell Signaling) b. phosphoERK (E-4, Santa Cruz). Blots were re-probed with an antibody for cyclophilin A (cyclo A) to ensure equal loading. c. Schematic of the MAP kinase cascade. U0216 inibits the MEK kinase and the protein downstream of MEK, ERK.


A.
α ERK
cyclo A
HGF - + - + - + - +
Minutes 10 20 30 60


B.
α pERK
cyclo A
HGF + + + + + + + +
U0126 - + - + - + - +
Minutes 10 20 30 60

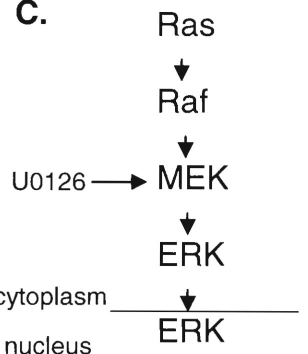
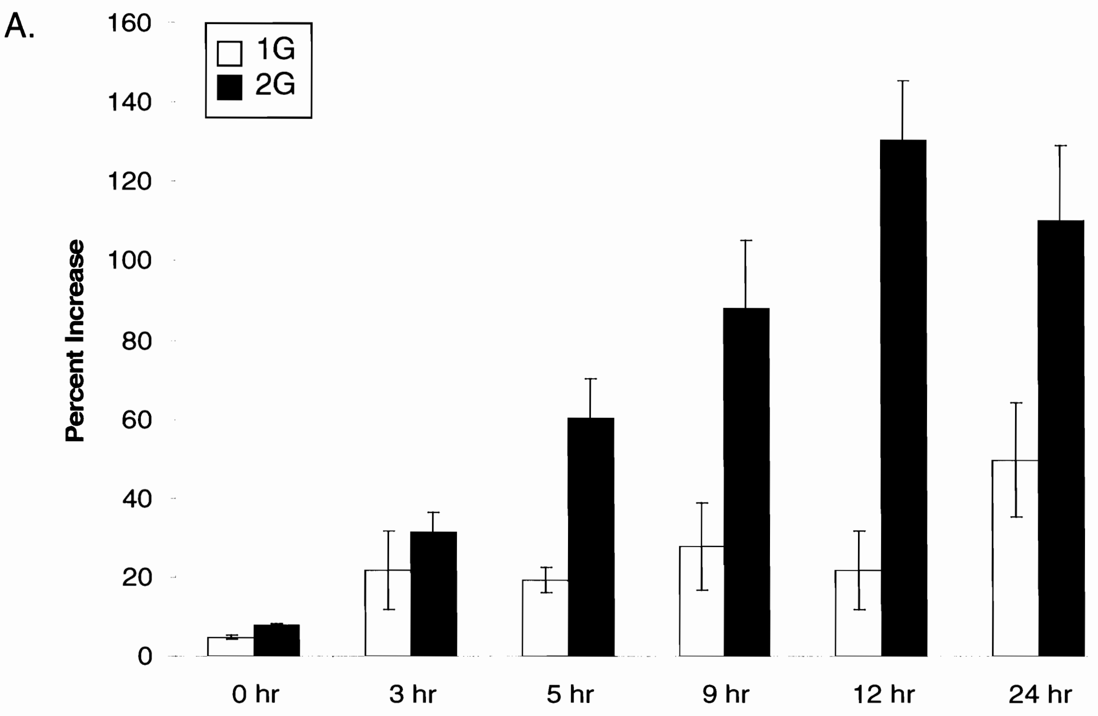
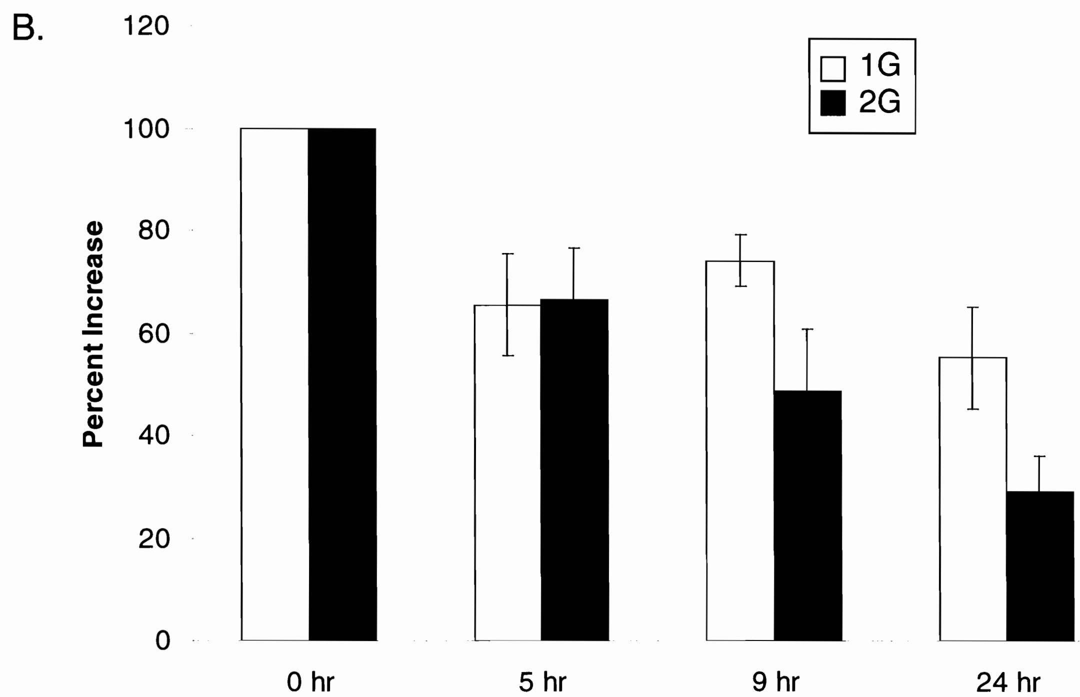

C.
Ras
Raf
U0126
MEK
ERK
cytoplasm
ERK
nucleus

Figure 15. Response of MMP-1 2G promoter to HGF is more marked when compared to the MMP-1 1G promoter response. a.U251 glioma cells were transiently transfected with 1ug of MMP-1 promoter DNA and 0.1 ug Renilla as an internal control. Cells were starved from serum overnight and treated with 30 ng/mL HGF for the indicated timepoint. Data is represented as percentage increase (minus HGF to plus HGF). Error bars indicate the SEM of four independent experiments. b. U251 glioma cells were transiently transfected with 1ug of MMP-1 promoter DNA and 0.1 ug Renilla as an internal control. Cells were starved from serum overnight and treated with 30 ng/mL HGF. Cells receiving inhibitor were treated with 10 uM U0126 thirty minutes prior to HGF treatment. Zero hour data is set to 100%. Data is represented as the percentage decrease of promoter activity in response to the addition of U0126 subtracted from the zero hour timepoint. Error bars indicate the SEM from three independent experiments.

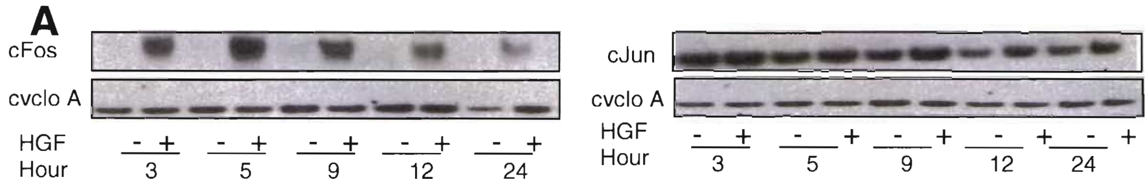

A.
160
140
120
100
80
60
40
20
0
Percent Increase
1G
2G
0 hr
3 hr
5 hr
9 hr
12 hr
24 hr

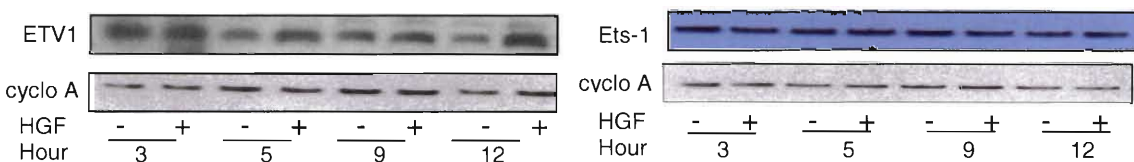

B.
120
100
80
60
40
20
0
Percent Increase
1G
2G
0 hr
5 hr
9 hr
24 hr

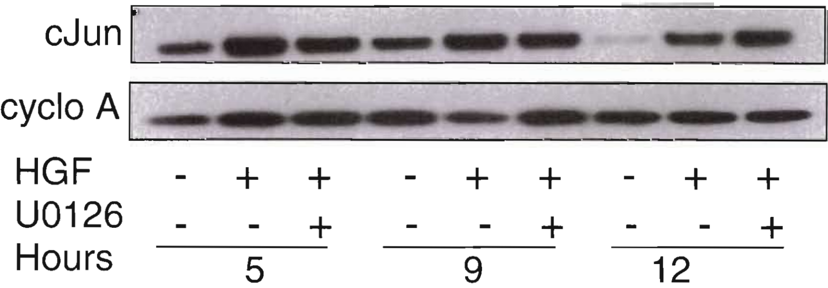
HGF/SF stimulates the induction of AP-1 proteins but not ETS proteins. The data from the luciferase assays indicated that MMP-1 2G promoter activity increases compared with the MMP-1 1G promoter activity approximately 5 hours after HGF/SF stimulation. Increases in promoter activity due to protein phosphorylation would presumably occur much sooner than 5 hours. Due to this delayed promoter activation, we examined the protein levels of transcription factors capable of binding to the distal MMP-1 promoter. These include the proteins in the ETS and AP-1 families. We stimulated U251 cells with HGF for various timepoints and performed western blot analysis on nuclear proteins (Figure 16a). The ETS family proteins Ets-1 and ETV1 remain unchanged in response to HGF/SF whereas the AP-1 family proteins, cJun and cFos are increased at 3 hours. The addition of U0126 inhibits the increase in the AP-1 protein levels (Figure 16b). This suggests that the increases in cJun and cFos are due to activation of the MAP kinase pathway.

ETS protein binding is dependent on glioma cell MMP-1 genotype. HGF/SF treatment not only affected both MMP-1 1G and 2G transcriptional activities but it also led to increases in protein levels of two AP-1 family members. We next wanted to determine if these proteins bind the MMP-1 distal promoter. We performed a chromatin immunoprecipitation assay (ChIP) to determine if these proteins bind the MMP-1 distal promoter (Figure 17). We treated the glioma cells with HGF/SF and performed the ChIP assay with antibodies for two AP-1 proteins (cJun and cFos) and two ETS proteins (Ets-1 and ETV1). We used primers designed to amplify the distal ETS and AP-1 binding sites (-1607 and -1602 respectively) to determine which proteins bind these sites in the MMP-1 promoter. The AP-1 proteins cFos and cJun bind both

Figure 16. AP-1 proteins are synthesized in response to HGF treatment in U251 cells. a.U251 glioma cells were treated with 30 ng/mL HGF for the indicated time. Nuclear proteins were extracted and 10 ug was used for a western blot. Top left: cFos (H125, Santa Cruz) Top right: cJun (H-79, Santa Cruz) Bottom left: ETV1 (custom made) Bottom right: Ets-1 (N-276, Santa Cruz) b.U251 glioma cells were treated with 30 ng/mL HGF for the indicated time. Cells receiving inhibitor were treated with 10 uM U0126 thirty minutes prior to HGF treatment. Nuclear proteins were extracted and 10 ug was used for a western blot. Top: cJun (H-79, Santa Cruz) Bottom: cFos (H125, Santa Cruz)


A
cFos
cvclo A
HGF
Hour
3
5
9
12
24
cJun
cvclo A
HGF
Hour
3
5
9
12
24


ETV1
cyclo A
HGF
Hour
3
5
9
12
Ets-1
cvclo A
HGF
Hour
3
5
9
12


B
cJun
cyclo A
HGF
U0126
Hours
5
9
12

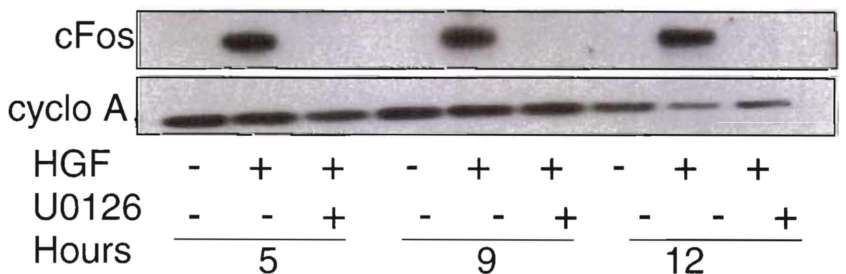
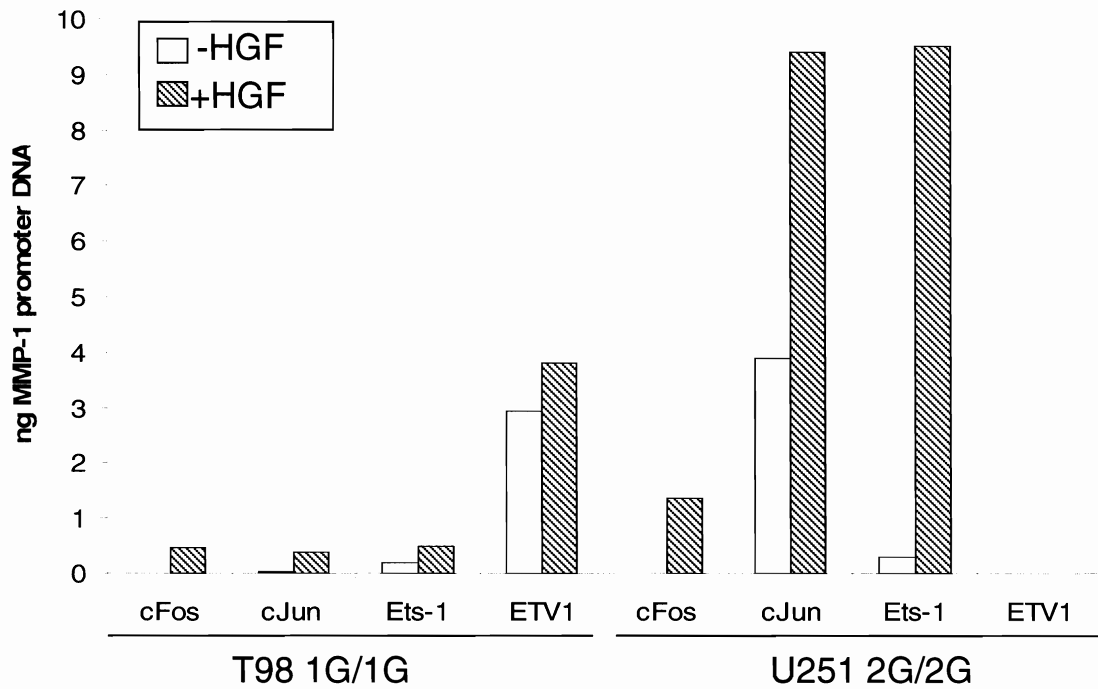

cFos
cyclo A
HGF
U0126
Hours
5
9
12

Figure 17. Binding pattern of ETS and AP-1 proteins to the 2G MMP-1 distal promoter is different than that of the 1G MMP-1 distal promoter. U251 and T98 glioma cells were treated 30 ng/mL HGF for 5 hours. The cells were then cross-linked with 1% formaldehyde, lysed, and proteins were immunoprecipitated with antibodies from the ETS and AP-1 transcription factor families. Primers were used to amplify the MMP-1 distal promoter using Sybr Green Real Time PCR. This graph is a representative experiment from three independent experiments.



-HGF
+HGF
ng MMP-1 promoter DNA
10
9
8
7
6
5
4
3
2
1
0
cFos
cJun
Ets-1
ETV1
cFos
cJun
Ets-1
ETV1
T98 1G/1G
U251 2G/2G

the 1G and 2G promoters after HGF/SF treatment, however the amount of cJun associated with the distal 2G MMP-1 promoter is higher. Treatment with HGF/SF also led to an increase in Ets-1 binding to the 2G MMP-1 promoter. The amount of cJun and Ets-1 proteins bound to both the 1G and 2G MMP-1 distal promoter is similar after the HGF/SF treatment. ETV1 binds only to the 1G promoter and this binding is not affected by HGF/SF. Treatment with the MEK inhibitor U1026 inhibits protein binding to both the 1G and 2G promoters (data not shown) indicating the binding may be dependent upon MAP kinase activation.

4.4 DISCUSSION

One of the reasons for the high mortality rate of gliomas is recurrence of the tumor due to the highly invasive nature of glioma cells. MMPs are key players in this invasive process in gliomas[113,115,123]. MMP-1 has not been as extensively studied in the brain as other proteases due to the current understanding of the role of MMP-1. That role is expanding to include much more than just the breakdown of collagen type I. MMP-1 can liberate growth factors from the ECM which can lead to dysregulation of signal transduction pathways and aberrant gene expression[105]. This role represents one potential mechanism for MMP-1 dependent increases in brain tumor invasion.

The results from this study indicate that HGF/SF stimulation of glioma cells leads to increases in MMP-1 mRNA levels. Glioma cells with the 2G/2G genotype have a more robust response to HGF/SF treatment than the cells containing the 1G/1G genotype. We propose that the extent of the response is dependent upon the genotype of the promoter although it is possible there are other mechanisms responsible for the increase in mRNA levels. The increase in MMP-1 mRNA levels could be due to an increase in promoter activity. Data from the luciferase reporter assays suggests that the increase in MMP-1 promoter activity in response to HGF/SF treatment is also genotype dependent. Glioma cells transiently transfected with the full length 1G promoter do not respond to HGF/SF treatment in the same manner as the glioma cells transiently transfected with the full length 2G promoter. The percentage increase of the 2G promoter is, on average, four fold greater than the increase of the 1G promoter.

It is not surprising that 1G promoter activity increases in response to HGF/SF treatment since the full length promoter used for the transfection assays has ETS and

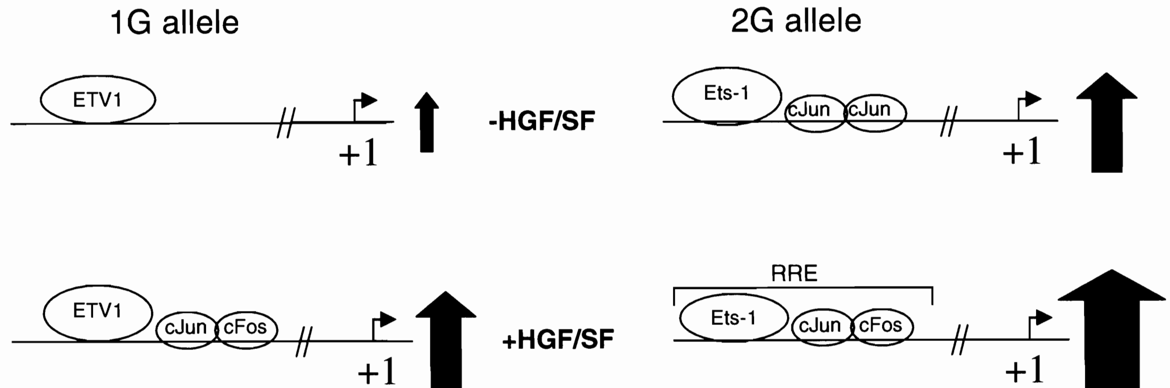
AP-1 binding sites in the proximal promoter region. Jinnin et al[94] determined that the proximal ETS binding site is critical for HGF responsiveness in fibroblasts. The one difference between the two promoters is that the 2G promoter has the additional guanine base, creating an ETS binding site. This consensus sequence is absent in the 1G full length promoter therefore the increased response of the 2G construct to HGF/SF stimulation may be attributed to this additional ETS binding site. The ETS binding site is adjacent to an AP-1 binding site fulfilling the requirements for a Ras Responsive Element (RRE). This element is responsible for synergistic upregulation of promoters involved in invasion and migration[124]. In order for the RRE to lead to synergistic upregulation, the ETS and AP-1 proteins must bind to the RRE in response to MAP kinase activation. Data from our studies indicates that the MAP kinase pathway is active in glioma cells leading to activation of the ERK kinase. Western blot analysis indicates that ERK activation increases the protein levels of AP-1 family members cJun and cFos, but does not increase the levels of the ETS proteins Ets-1 and ETV1. Prior to HGF/SF stimulation both glioma cells contain Ets-1, ETV1 and cJun proteins. After HGF/SF treatment, the levels of the ETS members remained unchanged, cJun levels increase slightly and cFos is induced in both T98 and U251 glioma cells. Our data suggests that the increase in MMP-1 transcription as a result of the ERK activation is due to cFos induction. Transcriptional activity of both the 1G and the 2G promoters begin to increase at approximately three hours after HGF/SF treatment. The increased response from the 2G promoter begins at approximately five hours. Western blot analysis suggests that this is the timepoint in which the glioma cells contain the highest levels of cFos. If the increase in promoter activity was due only as a result of a phosphorylation event, we would have expected the increase in

promoter activity to peak at a timepoint earlier than five hours. It could be that Ets-1 is phosphorylated in response to HGF/SF, as the literature suggests[91], and then is activated to bind to and activate the cFos promoter resulting in an increase of cFos protein[79]. The cFos, present in the nucleus, forms dimers with the pre-existing cJun. The cJun-cFos dimers are more stable than individual proteins and this leads to increased DNA binding activity[121].

Data from the ChIP assay indicates that HGF/SF affects protein binding in the MMP-1 distal promoter. In the absence of HGF/SF, similar proteins are bound in both 1G/1G cells and 2G/2G cells (Figure 18). cFos is not present in the nucleus prior to the addition of the HGF/SF and therefore not bound to the DNA in either the T98 (1G/1G) or the U251 cells (2G/2G). cJun is bound to both promoters in the absence of HGF/SF. There is more cJun bound to the 2G promoter than is bound to the 1G promoter prior to HGF/SF treatment. The addition of HGF/SF changes the binding pattern observed in the glioma cells. cFos is now available in the nucleus and binds to both promoters, presumably as a dimer with cJun. As with the cJun, there is more cFos bound to the 2G promoter than to the 1G promoter. In the 1G/1G glioma cells, there is a slight increase in Ets-1 binding after the growth factor treatment however in the 2G/2G glioma cells there is a much larger increase in Ets-1 binding after HGF/SF treatment.

It is somewhat surprising that even a small amount of Ets-1 binds to the 1G/1G promoter because that promoter does not contain the consensus binding site for the ETS proteins. The ability of proteins to bind to the endogenous distal promoter, either

Figure 18. Schematic of the proteins bound to the MMP-1 distal promoter as determined by chromatin immunoprecipitation.



1G allele
2G allele
ETV1
-HGF/SF
Ets-1
cJun
cJun
+1
+1
RRE
ETV1
cJun
cFos
+HGF/SF
Ets-1
cJun
cFos
+1
+1

1G/1G or 2G/2G has not been evaluated in any cell type to date. Electromobility shift assays conducted in melanoma cells indicate a similar binding pattern to both the 1G and 2G probes but the bands bound to the 2G probe were more intense. The authors suggest that the 1G ETS site may represent a weak binding site for proteins. This is supported by reports that indicate that ETS proteins can be rather promiscuous in binding to DNA[75]. Protein-protein interaction and phosphorylation events increase the strength of ETS binding to the promoters[75]. Since more Ets-1 protein is bound to the 2G promoter than the 1G promoter and the 2G promoter is more transcriptionally active, it suggests that the 2G ETS site is a true ETS binding site and therefore possibly responsible for increases in MMP-1 transcription in glioma cells.

Another striking difference in the binding pattern between the two promoters is that the ETV1 protein is bound only to the 1G promoter. This again is somewhat surprising in that the 1G promoter does not contain the consensus sequence for this protein. Although the core binding sequence for ETS proteins is GGAA/T, the specificity of binding to DNA is determined by the sequence flanking the core, protein-protein interaction and phosphorylation events[74]. It could be as in the case of the Ets-1 protein that the ETV1 binding to the 1G promoter represents a very weak interaction. This protein is a member of the PEA3 subfamily and as such it only retains 60% of its identity to Ets-1. It does not contain the pointed domain which is thought to be responsible for protein-protein interactions as well as phosphorylation by MAP kinase which docks onto the pointed domain. The mouse homolog ER81 can be activated by ERK, not by a direct phosphorylation event but perhaps through a protein downstream from ERK[125]. Data from our experiments indicate that in glioma cells ERK is affecting neither the protein levels of ETV1 nor its ability to bind to the 1G promoter. This

supports the theory that ETV1 is not phosphorylated by ERK. Currently there is little evidence to suggest that ETV1 interacts with AP-1 proteins to activate transcription. If either phosphorylation or protein interaction is required for a strong binding interaction, the criteria would not be met and ETV1 protein would be incapable of activating the promoter.

A second possibility is that ETV1 is binding to the 1G allele and repressing the promoter activity in the T98 cells, however there is no evidence to support this theory in the literature. We theorize that ETV1 is bound to the 1G promoter as a weak, perhaps transient binding event. In the gliomas cells with a 2G promoter, ETV1 cannot bind because the binding site is occupied with a multiprotein complex consisting of Ets-1, cJun, and cFos and the weakly binding ETV1 cannot replace this tightly bound complex.

The data presented herein reveals one possible mechanism for the difference in transcriptional activity between the 1G and 2G MMP-1 promoters in glioma cells. Understanding the importance of the polymorphism in the MMP-1 promoter and its influence on transcription of a gene thought to contribute to tumor invasion is an important step in uncovering the complex process of glioma recurrence.

4.5 MATERIALS AND METHODS

4.5.1 Cell Lines and Culture Conditions. T98 and U251 human glioma cells were obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin in a humidified incubator at 37° C with 5% CO_2. Cells were treated with 30ng/mL HGF (R&D Systems) for 5 hours. 10 µM U0126 (EMD Biosciences) was added 30 minutes prior to HGF treatment.

4.5.2 Genotyping and Sequencing. Genomic DNA was extracted from T98 and U251 glioma cells using the QiaAmp DNA Mini Kit (Qiagen) and quantified by spectrophotometry. Analysis of the SNP in the MMP-1 promoter was conducted on the 100 ng of DNA following the protocol as previously described [56]. The genotype of the gliomas and normal tissue was confirmed by selecting random samples and subjecting them to PCR previously described by Hirata *et al.* (2003) followed by direct DNA sequencing performed at the University of Iowa Sequencing Facility.

4.5.3 Immunoblotting. Ets-1 (N-276), cJun (H-79), cFos (H-125), cMet (C-12), and phospho-ERK (E-4) antibodies were purchased from Santa Cruz Biotechnology. Antibody for ETV1 was produced by PrimmBiotech using the peptide sequence published by the Jessell Laboratory (Columbia University). Nuclei were extracted [104] and quantified using the Coomassie Plus Protein Assay (Pierce). 10 µg of protein was loaded into a 4-12 % Bis Tris Gel and a Western Blot was performed using the antibodies described above.

4.5.4 Chromatin immunoprecipitation. T98 (1G/1G) and U251 (2G/2G) glioma cells were treated with 30 ng/mL HGF for 5 hours. Samples receiving U0126 (10µM) were treated for 30 minutes prior to HGF treatment. Formaldehyde (Fisher Scientific) was

added at a final concentration of 1% directly to cell culture media for 8 minutes to crosslink the bound proteins to DNA. Cells were collected, washed and lysed by sonication so that the average chromatin length was less than 1 kb. Samples were precleared with Protein A agarose beads (Roche) for 1 hour rotating at 4°C and then incubated overnight with 1μg of antibody. Protein A agarose beads were added for 1 hour, samples were washed and protein-DNA complexes were eluted from the beads (2% SDS, 10mM DTT, 0.1M $NaHCO_3$). The crosslinking was reversed with 0.2M NaCl at 65°C for 4 hours, proteins were removed by phenol-chloroform extraction and DNA was precipitated and quantitated by spectrophotometry. Sybr Green (Qiagen) Real Time PCR was performed with primers for both proximal and distal regions of the MMP-1 promoter. Primer sequences were as follows: proximal [126]; distal forward 5'-CAGTGGCAAGTGTTCTTTGG-3'; distal reverse 5'-CTCCCACCTTTCCCACTGTA-3'.

4.5.5 Transfection and Luciferase assays. Cells were transiently transfected in triplicate using the LipofectAMINE 2000 reagent (Invitrogen) with 1 μg of MMP-1 promoter/reporter plasmids (a kind gift from Dr. Constance Brinckerhoff) and 2 μL LipofectAMINE 2000. U0126 and HGF treatments began 48 hours following the transfection. Cells were lysed with reagents from the Dual Luciferase Reporter Assay Kit (Promega). Luciferase activity was measured on the FLUOstar 403 (BMG Lab Technologies) Samples were normalized to the internal control plasmid Renilla which was concurrently transfected into the cells with the MMP-1 promoter.

4.5.6 RNA Extraction and Real Time PCR. Total RNA was extracted from T98 and U251 human glioma cells by following the TRIzol protocol supplied by the manufacturer (Invitrogen). RNA was quantified using spectrophotometry and sent to the Molecular Core of the Virginia Commonwealth University-Massey Cancer Center Nucleic Acids

Research Facilities for Real Time PCR analysis of MMP-1 mRNA levels using the ABI 7900 Sequence Detection System.

Chapter 5

Discussion

The data presented herein strongly suggests that the polymorphism in the MMP-1 promoter is important in glioma biology. In the first half of this thesis we presented data on the characterization of the polymorphism in the MMP-1 promoter. There was a statistically significant increase in the percentage of the 2G/2G genotype in patients with a GBM when compared to the control population. This signifies that the polymorphism must have some role in glioma biology. It would seem unlikely that the 2G allele, which generates a higher level of MMP-1 promoter activity, would be present in a higher proportion of tumor patients if the additional activity did not contribute, in some way, to the biology of the tumor. In the MMP-1 regulatory section, I stated that the main level of regulation for this enzyme was at the transcriptional level. This is another piece of evidence suggesting that the allele with a higher activity level would not be present in greater percentages without fulfilling some role in the biology of the tumor. We did not investigate any possible roles in my project but merely characterized the SNP and began an investigation of one possible mechanism of action responsible for the increased transcription from the 2G promoter.

I think it is important to take a step back from my project and understand the function of MMP-1 in brain tumors. Currently there is no known role for MMP-1 in brain tumors and there are only three papers[44-46] (one of which is from our laboratory) that have assessed the amount of MMP-1 in gliomas. If I were to study the function of MMP-1 in gliomas, I would first review the roles that MMP-1 has in both the normal brain and in other disease states. Based on the information presented in the introduction chapter of this thesis, it is clear that MMP-1 is present in the brain, but there is question a as to which cells are secreting the enzyme. It could be that the glioma cells themselves are not secreting the enzyme but are affected by the MMP-1

secreted by cells in the stroma. If MMP-1 is present in the brain, regardless of which cells it is secreted from, the effect of the polymorphism in gliomas should be studied. The cell with a 2G/2G genotype will transcribe more MMP-1 than a cell with a 1G/1G genotype, be it normal or tumor cell. It is important to identify potential roles for MMP-1 in gliomas since the 2G/2G genotype was significantly higher in glioma patients than in control individuals.

Since the polymorphism leads to increases in MMP-1 transcription it is assumed that the transcribed mRNA will become protein. One would expect the cells with the 2G/2G genotype to express the most MMP-1 protein, the cells with the 1G/2G genotype to express less than the 2G/2G and the cells with the 1G/1G genotype to express the least amount of MMP-1. This was not the case in our study[44] and if one reads the literature carefully it may not be the case in the other studies[49,55,59] . The groups that report a correlation between genotype and MMP-1 protein levels combine the 1G/2G and 2G/2G samples when analyzing MMP-1 protein levels. This is interesting for two reasons: 1) the cells with the highest level of MMP-1 transcription may not have the highest level of MMP-1 protein and 2) the cells heterozygous for the MMP-1 polymorphism (1G/2G) have the highest levels of MMP-1 protein. In our cell lines the basal level of MMP-1 mRNA does not correspond to genotype but cell lines with a 2G/2G promoter respond more robustly to HGF/SF treatment. Of course one needs to consider that as in all glioma tumors, glioma cell lines differ in more ways than the MMP-1 promoter polymorphism. To circumvent this problem, one could use an isogenic line for the experiments.

After characterizing the polymorphism in the MMP-1 promoter in glioma tissues, I explored a potential mechanism for the increase in transcription due to the presence of the additional guanine nucleotide in the 2G promoter. The information taken from this data may also help us elucidate a possible functional mechanism for the polymorphism by pinpointing molecular pathways to target. The data indicates that HGF/SF treatment of glioma cell lines leads to increases in not only MMP-1 transcription but also MMP-1 mRNA in a genotype dependent manner. Data from the EMSA experiment indicates that proteins are capable of binding to a probe mimicking the 2G promoter. I was unable to identify specific proteins through a supershift assay however the competition assays suggest that Ets-1 binds to the probe. I performed the EMSA with a probe corresponding to the 1G promoter (not shown) and the binding pattern was identical to that of the 2G probe. This is in agreement with the data published by Rutter *et al.*[47] who suggested that similar proteins were binding to both probes but that the 2G probe was a "better" binding site due to the additional guanine nucleotide. To determine if the same proteins are binding to both the 1G and the 2G promoter probes, one could repeat the EMSA using the 2G probe and use an unlabeled 1G probe as a competitor. If the band corresponding to the proteins binding to the 2G probe is competed by the 1G probe that will indicate that the same proteins are binding to both probes. Based on the data from the ChIP assay it would seem unlikely that the same proteins are binding to both probes however the EMSA is performed using an artificial probe and nuclear extracts. The ChIP assay is performed while the cells are growing in culture and the nuclei are intact and therefore the chromatin is intact and if chromatin structure is necessary for protein dimerization or binding, it would not occur in the EMSA. I would also repeat the EMSAs using nuclear

extracts isolated from HGF/SF treated cells to determine if the growth factor stimulation causes increased binding or changes the binding pattern in any way. Based on the data from the ChIP assay I would suspect that there would be additional bands corresponding to either cFos alone or cFos as a part of a protein complex. It would be interesting to see if the proteins need intact chromatin for binding. This could implicate histone modifications such as acetylation and perhaps other necessary proteins such as CBP/p300 as a requirement for MMP-1 promoter activation.

I think it would be valuable to determine if the addition of HGF/SF increases the amount of active MMP-1 in the glioma cells that contain the 2G/2G promoter. I demonstrated that the promoter activity increases and the mRNA levels increase after HGF/SF treatment and that the cells with a 2G/2G promoter have a more robust response to HGF/SF but never tested the effect on increases in protein or active enzyme. I acknowledge that I addressed this point of increased promoter activity leading to increases in active enzyme in the beginning of this discussion section but I think it is very important to distinguish between an active promoter which may not have any effect on the cell and active enzyme in the cell which may contribute to tumor invasion, recurrence or both. Since there are many growth factors present in the tumor environment it would be important to study this phenomenon in the presence of the growth factor as well.

Addition of the HGF/SF causes an increase in protein levels of cJun and cFos, while the protein levels of the ETS members did not change in response to HGF/SF. It could be that the HGF/SF activated the MAP kinase cascade which causes the phosphorylation of the ETS proteins. I investigated this possibility with three different

methods but each experiment gave results that were inconclusive. Initially I tried immunoprecipitation of Ets-1 protein from HGF/SF stimulated cells. I then ran the protein that was removed from the beads on a western blot and probed with an antibody specific for phospho-threonine. The secondary antibody used to detect the phospho-threonine antibody cross-reacted with the Ets-1 antibody used in the immunoprecipitation step because they were both raised in a rabbit. Since the heavy chain of the IgG is 55 kDa and the Ets-1 protein is 54 kDa this cross reaction prevented me from seeing if there was a band in the lane corresponding to the HGF/SF treated cells because the heavy chain was quite a substantial size. To circumvent this problem, we tried using a new product from Molecular Probes that stained the gel itself so there is no need for secondary antibody. In this experiment, the results between the stimulated and unstimulated samples were the same indicating to us that Ets-1 was not phosphorylated as a result of the HGF/SF treatment. We lacked a convincing positive control so we were not sure if the method was working and the samples were indeed phosphorylated.

The question of whether or not Ets-1 is phosphorylated should be addressed. The literature contains data suggesting that Ets-1 is phosphorylated as a result of HGF/SF treatment however the data was based on in vitro kinase assays. This indicates that Ets-1 contains the phosphoacceptor site and therefore could be phosphorylated but no one has tried to immunoprecipitate Ets-1 from cells and determine the phosphorylation status. I recommend using an Ets-1 antibody that has been commercially biotinylated to prevent any ambiguity about the biotinylation efficiency. This method would cost more, but this would, in theory, prevent the cross reaction between the Ets-1 antibody used in the immunoprecipitation step and the

secondary antibody heavy chain used on the western blot. The problem of the positive control still remains, but there are a few imperfect controls one could use. One would be to use an antibody for phosphoERK on an additional sample since, based on the evidence presented in chapter 4, we know ERK is phosphorylated in response to HGF/SF treatment. One problem with that control is that if there is a problem with the Ets-1 antibody we would not know so one will have to show that the immunoprecipitation is working each time. This, combined with the phosphoERK control would control for both the HGF/SF treatment working properly and control for the Ets-1 immunoprecipitation. Another potential control would be to stimulate cells with a different growth factor known to phosphorylate Ets-1. This would test the phosphorylation detection method used. I would also test various timepoints to see when the protein is phosphorylated and how this correlates with increases in cFos and cJun protein levels and MMP-1 promoter activity. The genotype of the cell should not have any influence on the phosphorylation status of Ets-1, so it will not matter which cell line one uses.

ETV1 was the other ETS member we investigated with respect to protein levels after HGF/SF stimulation and binding capability. The data from the Western blots on extracts stimulated with HGF/SF is similar to that of Ets-1; there is no increase in protein levels following HGF/SF at any of the timepoints we tested. It is unlikely that ETV1 is phosphorylated in response to HGF/SF, as we propose Ets-1 is, because ETV1 does not contain the same structural element, the pointed domain, needed for phosphorylation by MAP kinase. Data from the ChIP assay indicates that ETV1 is bound only to the 1G/1G promoter. This could indicate that when ETV1 is bound to the promoter it is somehow repressing promoter activity either by interfering with binding of

Ets-1 or perhaps interfering with AP-1 proteins binding to the promoter. One could perform experiments to address these possibilities. I would clone the cDNA for ETV1 into an expression vector, transfect the vector into glioma cells and then perform luciferase promoter assays with the MMP-1 reporter constructs. If the promoter activity of the cells with the additional ETV1 decreased in comparison with those cells lacking the additional ETV1 it could indicate a potential repressive activity. I would follow up that experiment with one in which the ETV1 is removed from the cell with either siRNA or antisense technology. In this case the promoter in the cells with the lesser amount of ETV1 should have a higher level of MMP-1 1G promoter activity if the ETV1 is indeed repressing MMP-1 transcription. To determine if ETV1 is somehow interfering with AP-1 protein binding I would use the same approach as I just described but instead perform a ChIP assay to see if the cells with the overexpressed ETV1 have less AP-1 binding. I would also perform co-immunoprecipitation studies to determine if ETV1 is directly bound to the AP-1 proteins.

In addition, co-immunoprecipitation studies performed with Ets-1 and AP-1 proteins as well, could provide information on the physical interactions of those two proteins. If the results from the co-immunoprecipiation studies indicate there is no physical interaction, it does not mean that they are not interacting on the promoter DNA. If they do interact in solution however, it would be powerful data to support my hypothesis that cooperation between ETS and AP-1 is one possible mechanism responsible for the increase in the 2G MMP-1 promoter in response to HGF/SF treatment.

Prior to HGF/SF treatment, cFos is not present in the glioma cell lines. HGF/SF treatment causes an increase in the levels of the cFos protein and also causes an increase in MMP-1 promoter activity and mRNA levels. One of our hypotheses is that the presence of cFos, as a result of the HGF/SF treatment, creates an AP-1 dimer, composed of cJun and cFos, which represents the protein with the highest capability to stimulate MMP-1 transcription. The MMP-1 promoter with the highest level of activity is the promoter that has Ets-1 bound in cooperation with cJun and cFos. To test this hypothesis, one could knockdown the cFos in the glioma cells with either siRNA or antisense treatments. After performing the necessary experiments to determine that the knockdown is working I would repeat the ChIP assay and determine if cFos is still binding. The ChIP assay would also help determine if cFos is necessary for the other proteins to bind. I would repeat the promoter studies to determine if the cFos is necessary for increases in MMP-1 transcription. If the cooperation between AP-1 and Ets-1 is necessary for promoter stimulation, then the difference in activity levels between the 1G and the 2G construct should diminish. If the cooperation is unnecessary, then both promoters should decrease similarly.

In the long term it will be important to link this polymorphism with a functional role in gliomas. I think that by first studying the role of MMP-1 in gliomas, the possible roles can be narrowed down. Based on the function of MMP-1 in other pathological states, potential roles for this polymorphism could include: 1) a contributor to treatment resistance, 2) increased susceptibility to gliomas 3) increased aggressiveness of the tumor and 4) increased inflammatory response. Preliminary data from our laboratory suggests that the overexpression of MMP-1 in one glioma cell line results in not only invasion but also cell growth as measured by colony forming assays. Members of our

laboratory have also begun invasion assays on two glioma cell lines with different MMP-1 promoter genotype. It will be interesting to determine if the invasiveness of the glioma cell lines can be correlated to the MMP-1 promoter status. There is evidence that MMP-1 is involved in apoptosis, both pro and anti-apoptotic as mentioned in the introduction. I would perform experiments to evaluate this role of MMP-1 with respect to the polymorphism status in the distal promoter.

One last thing I would look at to evaluate a functional role for this polymorphism is the possible relationship between the MMP-1 genotype and treatment resistance. There is data in the literature linking p53 to decreases in MMP-1 transcription through a mechanism that interferes with AP-1 binding to the proximal promoter[67-69]. It is well known that irradiation treatment stabilizes the half life of wild type p53. In cells with intact p53, irradiation will theoretically decrease the levels of MMP-1 transcription. Perhaps, in cells with a 2G/2G promoter, because they have higher basal and stimulated levels of MMP-1, the p53 cannot repress the promoter as well as in cells with a 1G/1G promoter. The effect of irradiation in cells with a 2G/2G promoter may not be as great as on cells with a 1G/1G promoter and this will lead to differences in response to treatment and ultimately patient outcome. This is just one example of a potential functional consequence of the polymorphism in the MMP-1 promoter. It has not been analyzed in any detail and therefore is purely speculative.

It is clear that the distribution of the polymorphism in the MMP-1 promoter is different in glioma patients than in controls suggesting an importance in glioma biology. Through experiments presented in this discussion and thoughtful discussion of the results from these experiments, we may have a clearer understanding of not only the

mechanism behind the increase in MMP-1 transcription resulting from the 2G promoter but we may also begin to understand the role of this polymorphism in gliomas. It is this understanding that will be necessary for researchers to design therapeutics to combat this deadly disease.

REFERENCES

REFERENCES

1. McKinney P. Brain Tumours: Incidence, Survival, and Aetiology. *Journal of Neurology, Neurosurgery and Psychiatry* **75**:ii12-ii17 (2004).

2. Behin A, Hoang-Xuan K, Carpentier A & Delattre J-Y. Primary brain tumours in adults. *The Lancet* **361**:323-331 (2003).

3. Collins V. Brain Tumours: Classification and Genes. *Journal of Neurology, Neurosurgery and Psychiatry* **75**:ii2-ii11 (2004).

4. Maher E, Furnari F, Bachoo R, Rowitch D, Louis D, Cavenee W & DePinho R. Malignant glioma: genetics and biology of a grave matter. *Genes Dev.* **15**:1311-1333 (2001).

5. CBTRUS. Statistical Report: Primary Brain Tumors in the United States. 2005.

6. Mehta M, Gilbert M, Mirimanoff R & Brown P. Is Combination Chemoradiation the New Gold Standard for gliomas. *Medical Communications Media* (2004).

7. Mitchell P, Ellison D & Mendelow A. Surgery for malignant gliomas: mechanistic reasoning and slippery statistics. *Lancet Neurology* **4**:413-422 (2005).

8. Vitaz T, Warnke P, Tabar V & Gutin P. Brachytherapy for brain tumors. *Journal of Neuro-Oncology* **73**:71-86 (2005).

9. Lonardi S, Tosoni A & Brandes A. Adjuvant chemotherapy in the treatment of high grade gliomas. *Cancer Treatment Reviews* **31**:79-89 (2005).

10. Carpentier A, Laigle-Donadey F, Zohar S, Capelle L, Behin A, Tibi I, Martin-Duverneuil N, Sanson M, Lacomblez L, Taillibert S, Puybasset L, Van Effenterre R, Delattre J-Y & Carpentier A. Phase I trial of a CpG oligonucleotide for patients with recurrent glioblastoma. *Neuro-Oncology* **8**:60-66 (2006).

11. Oldfield E, Broaddus W, Bruce J, Trask T, Laske D, McDonald J, Patel S, Weingart J, Wharen R & Youle R. Phase II trial of convection-enhanced distribution of recombinant immunotoxin in patients with recurrent malignant gliomas. *American Association of Neurological Surgeons Annual Meeting* (2000).

12. Parney I, Kunwar S, McDermott M, Berger M, Prados M, Cha S, Croteau D, Puri R & Chang S. Neuroradiographic changes following convection-enhanced delivery of the recombinant cytotoxin interleukin 13-PE38QQR for recurrent malignant glioma. *Journal of Neurosurgery* **102**:267-275 (2005).

13. Lidar Z, Mardor Y, Jonas T, Pfefer R, Faibel M, Nass D, Hadani M & Ram Z. Convection-enhanced delivery of paclitaxel for the treatment of recurrent malignant glioma: a phase I/II clinical study. *Journal of Neurosurgery* **100**:472-479 (2004).

14. Sampson J, Akabani G, Archer G, Bigner D, Berger M, Friedman A, Friedman H, Herndon II J, Kunwar S, Marcus S, McLendon R, Paolino A, Penne K, Provenzale J, Quinn J, Reardon D, Rich J, Stenzel T, Tourt-Uhlig S, Wikstrand C, Wong T, Williams R, Yuan F, Zalutsky M & Pastan I. Progress Report of a Phase I Study of the Intracerebral Microinfusion of a Recombinant Chimeric Protein Composed of Transforming Growth Factor (TGF)-α and a Mutated form of the *Pseudomonas* Exotoxin Termed PE-38 (TP-38) for the Treatment of Malignant Brain Tumor. *Journal of Neuro-Oncology* **65**:27-35 (2003).

15. Zhu Y & Parada L. The Molecular and Genetic Basis of Neurological Tumours. *Nature Reviews Cancer* **2**:616-626 (2002).

16. Brinckerhoff C & Matrisian L. Matrix metalloproteinases: a tail of a frog that became a prince. *Nature Reviews. Molecular cell biology* **3**:207-214 (2002).

17. Overall C & Lopez-Otin C. Strategies for MMP Inhibition in Cancer: Innovations for the Post-Trial Era. *Nature Reviews Cancer* **2**:657-672 (2002).

18. Brinckerhoff C, Rutter J & Benbow U. Interstital Collagenases as Markers of Tumor Progression. *Clinical Cancer Research* **6**:4823-4830 (2000).

19. Egeblad M & Werb Z. New Functions for the Matrix Metalloproteinases in Cancer Progression. *Nature Reviews Cancer* **2**:161-174 (2002).

20. Nagase H & Woessner Jr J. Matrix Metalloproteinases. *The Journal of Biological Chemistry* **274**:21491-21494 (1999).

21. Sternlicht M & Werb Z. How Matrix Metalloproteinase Regulate Cell Behavior. *Annual Review of Cell and Developmental Biology* **17**:463-516 (2001).

22. Vu T & Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* **14**:2123-2133 (2000).

23. Behrendtsen O & Werb Z. Metalloproteinases regulate parietal endoderm differentiating and migrating in cultured mouse embryos. *Develop Dyn* **208**:205-265 (1997).

24. Fisher C, Gilbertson-Beadling S, Powers E, Petzold G, Poorman R & Mitchell M. Interstitial collagenase is required for angiogenesis in vitro. *Developmental Biology* **162**:499-510 (1994).

25. Sato T, Foged N & Delaisse J. The migration of purified osteoclasts through collagen is inhibited by matrix metalloproteinase inhibitors. *Journal of Bone Mineral Research* **13**:59-66 (1998).

26. Koshikawa N, Giannelli G, Cirulli V, Miyazaki K & Quaranta V. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *Journal of Cell Biology* **148**:615-624 (2000).

27. Miralles F, Battelino T, Czernichow P & Scharfmann R. TGF-beta plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2. *Journal of Cell Biology* **143**:827-836 (1998).

28. Whitelock JM, Murdoch AD, Iozzo RV & Underwood PA. The Degradation of Human Endothelial Cell-derived Perlecan and Release of Bound Basic Fibroblast Growth Factor by Stromelysin, Collagenase, Plasmin, and Heparanases. *J. Biol. Chem.* **271**:10079-10086 (1996).

29. Manes S, Llorente M, Lacalle RA, Gomez-Mouton C, Kremer L, Mira E & Martinez A. The Matrix Metalloproteinase-9 Regulates the Insulin-like Growth Factor-triggered Autocrine Response in DU-145 Carcinoma Cells. *J. Biol. Chem.* **274**:6935-6945 (1999).

30. Manes S, Mira E, Barbacid MdM, Cipres A, Fernandez-Resa P, Buesa JM, Merida I, Aracil M, Marquez G & Martinez A. Identification of Insulin-like Growth Factor-binding Protein-1 as a Potential Physiological Substrate for Human Stromelysin-3. *J. Biol. Chem.* **272**:25706-25712 (1997).

31. Pyo R, Lee J, Shipley J, Curci J & Mao D. Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) supresses development of experimental abdominal aortic aneurysms. *Journal of Clinical Investigations* **105**:1641-1649 (2000).

32. Alexander CM, Howard EW, Bissell MJ & Werb Z. Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. *J. Cell Biol.* **135**:1669-1677 (1996).

33. Powell W, Fingleton B, Wilson C, Boothby M & Matrisian L. The metalloproteinase matrilysin proteolytically generates active soluble Fas Ligand and potentiates epithelial cell apoptosis. *Current Biology* **9**:1441-1447 (1999).

34. Yu WH, Woessner JF, Jr., McNeish JD & Stamenkovic I. CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. *Genes Dev.* **16**:307-323 (2002).

35. Baker AH, Edwards DR & Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* **115**:3719-3727 (2002).

36. Pardo A & Selman M. MMP-1: the elder of the family. *The International Journal of Biochemistry & Cell Biology* **37**:283-288 (2005).

37. Kouwenhoven M, Ozenci V, Gomes A, Yarilin D, Giedraitis V, Press R & Link H. Multiple sclerosis: elevated expression of matrix metalloproteinases in blood monocytes. *Journal of Autoimmunity* **16**:463-370 (2001).

38. Bar-Or A, Nuttall R, Duddy M, Alter A, Kim H, Ifergan I, Pennigton C, Bourgoin P, Edwards D & Yong V. Analyses of all matrix metalloproteinase members in leukocytes emphasize monocytes as major inflammatory mediators in multiple sclerosis. *Brain* **126**:2749 (2003).

39. Leake A, Morris C & Whateley J. Brain matrix metalloproteinase-1 levels are elevated in Alzheimer's disease. *Neuroscience Letters* **291**:201-203 (2000).

40. Sudbeck B, Pilcher B, Welgus H & Parks W. Induction and repression of collagenase-1 by keratinocytes is controlled by distinct components of different extracellular matrix compartments. *The Journal of Biological Chemistry* **272**:22103-22110 (1997).

41. Lorenzi S, Albers D, Chirichigno J, Augood S & Beal M. Elevated levels of matrix metalloproteinases-9 and -1 and of tissue inhibitors of MMPs, TIMP-1 and TIMP-2 in postmortem brain tissue of progressive supranuclear palsy. *Journal of the Neurological Sciences* **218**:39-45 (2004).

42. Vos C, Sjulson L, Nath A, McArthur J, Pardo C, Rothstein J & Conant K. Cytotoxicity by Matrix Metalloproteinase-1 in Organotypic Spinal Cord and Dissociated Neuronal Cultures. *Experimental Neurology* **163**:324-330 (2000).

43. Conant K, St.Hillaire C, Nagase H, Visse R, Gary D, Haughey N, Anderson C, Turchan J & Nath A. Matrix Metalloproteinase 1 Interacts with Neuronal Integrins and Stimulates Dephosphorylation of Akt. *The Journal of Biological Chemistry* **279**:8056-8062 (2004).

44. McCready J, Broaddus W, Sykes V & Fillmore H. Assiociation of a single nucleotide polymorphism in the matrix metalloproteinase-1 promoter with glioblastoma. *International Journal of Cancer* **117**:781-785 (2005).

45. Nakano A, Tani E, Miyazaki K, Yamamoto Y & Furuyama J-I. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gliomas. *Journal of Neurosurgery* **83**:298-307 (1995).

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

47. Rutter J, Mitchell T, Buttice G, Meyers J, Gusella J, Ozelius L & Brinckerhoff C. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. *Cancer Research* **58**:5321-5325 (1998).

48. Hirata H, Naito K, Yoshihiro S, Matsuyama H, Suehiro Y & Hinoda Y. A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 Promoter is associated with Conventional Renal Cell Carcinoma. *International Journal of Cancer* **106**:372-374 (2003).

49. Nishioka Y, Kobayashi K, Sagae S, Ishioka S, Nishikawa A, Matsushima M, Kanamori Y, Minaguchi T, Nakamura Y, Tokino T & Kudo R. A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 Promoter in Endometrial Carcinomas. *Japanese Journal of Cancer Research* **91**:612-615 (2000).

50. Noll W, Belloni D, Rutter J, Storm C, Schned A, Titus-Ernstoff L, Ernstoff M & Brinckerhoff C. Loss of Heterozygostiy on Chromosome 11q22-23 in Melanoma is Associated with Retention of the Insertion Polymorphism in the Matrix Metalloproteinase-1 Promoter. *American Journal of Pathology* **158**:691-697 (2002).

51. Dickey I & Scully S. Identification of a Single Nucleotide Polymorphism in the MMP-1 Promoter in Chondrosarcoma. *Journal of Surgical Oncology* **87**:130-133 (2004).

52. Lin S-C, Chung M-Y, Huang J, Shieh T-M, Liu C-J & Chang K-W. Correlation between functional genotypes in the matrix metalloproteinases-1 promoter and risk of oral squamous cell carcinomas. *Journal of Oral Pathological Medicine* **33**:323-326 (2004).

53. Ye S, Dhillon S, Turner S, Bateman A, Theaker J, Pickering R, Day I & Howell W. Invasiveness of Cutaneous Malignant Melanoma Is Influenced by Matrix Metalloproteinase 1 Gene Polymorphism. *Cancer Research* **61**:1296-1298 (2001).

54. Wenham R, Calingaert B, Ali S, McClean K, Whitaker R, Bentley R, Lancaster J, Schildkraut J, Marks J & Berchuck A. Matrix Metalloproteinase-1 Gene Promoter Polymorphism and Risk of Ovarian Cancer. *Journal of the Society for Gynecological Investigations* **10**:381-387 (2003).

55. Kanamori Y, Matsushima M, Minaguchi T, Kobayashi K, Sagae S, Kudo R, Terakawa N & Nakamura Y. Correlation between Expression of the Matrix

Metalloproteinase-1 Gene in Ovarian Cancers and an Insertion/Deletion Polymorphism in its promoter region. *Cancer Research* **59**:4225-4227 (1999).

56. Zhu Y, Spitz M, Lei L, Mills G & Wu X. A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 Promoter Enhances Lung Cancer Susceptibility. *Cancer Research* **61**:7825-7829 (2001).

57. Ghilardi G, Biondi M, Mangoni J, Leviti S, DeMonti M, Guagnellini E & Scorza R. Matrix Metalloproteinase-1 Promoter Polymorpshim 1G/2G Is Correlated with Colorectal Cancer Invasiveness. *Clinical Cancer Research* **7**:2344-2346 (2001).

58. Przybylowska K, Zielinska J, Zadrozny M, Krawcyzk T, Kulig A, Wozniak P, Rykala J, Kolacinska A, Morawiec Z, Drzewoski J & Blasiak J. An Association Between the Matrix Metalloproteinase 1 Promoter Gene Polymorphism and Lymphnode Metastasis in Breast Cancer. *Journal of Experimental Clinical Cancer Research* **23**:121-125 (2004).

59. Nishioka Y, Sagae S, Nishikawa A, Ishioka S & Kudo R. A Relationship between Matrix Metalloproteinase-1 (MMP-1) promoter polymorphism and cervial cancer progression. *Cancer Letters* **200**:49-55 (2003).

60. Fujimoto T, Parry S, Urbanek M, Sammel M, Macones G, Kuivaniem H, Romero R & Strauss III J. A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 (MMP-1) Promoter Influences Amnion Cell MMP-1 Expression and Rish for Preterm Premature Rupture of the Fetal Membranes. *The Journal of Biological Chemistry* **277**:6296-6302 (2002).

61. Wyatt C, Coon C, Gibson J & Brinckerhoff C. Potential for the 2G Single Nucleotide Polymorphism in the Promoter of Matrix Metalloproteinase to Enhance Gene Expression in Normal Stromal Cells. *Cancer Research* **62**:7200-7202 (2002).

62. Vincenti M, White L, Schroen D & Brinckerhoff C. Regulating Expression of the Gene for the Matrix Metalloproteinase-1 (Collagenase): Mechanisms that Control Enzyme Activity, Transcription and mRNA Stability. *Critical Reviews in Eukayotic Gene Expression* **6**:391-411 (1996).

63. Westermarck J, Seth A & Kahari V. Differential regulation of interstitial collagenase (MMP-1) gene expression by ETS transcription factors. *Oncogene* **14**:2651-2660 (1997).

64. White L, Maute C & Brinckerhoff C. ETS sites in the promoters of the matrix metalloproteinases collagenase (MMP-1) and stromelysin (MMP-3) are auxiliary elements that regulate basal and phorbol-induced transcription. *Connective Tissue Research* **36**:321-335 (1997).

65. Benbow U, Schoenermark M, Mitchell T, Rutter J, Shimokawa K, Nagase H & Brinckerhoff C. A Novel Host/Tumor Cell Interaction Activates Matrix Metalloproteinase 1 and Mediates Invasion through Type I Collagen. *The Journal of Biological Chemistry* **274**:25371-25378 (1999).

66. Benbow U, Schoenermark M, Orndorff K, Givan A & Brinckerhoff C. Human breast cancer cells activate procollagenase-1 and invade type I collagen: invasion is inhibited by all-trans retinoic acid. *Clinical & Experimental Metastasis* **17**:231-238 (1999).

67. Sun Y, Sun Y, Wenger L, Rutter J, Brinckerhoff C & Cheung H. P53 Down-regulates Human Matrix Metalloproteinase-1 (Collagenase-1) Gene Expression. *The Journal of Biological Chemistry* **274**:11535-11540 (1999).

68. Sun Y, Sun Y, Wenger L, Rutter J, Brinckerhoff C & Cheung H. Human Metalloproteinase-1 (Collagenase-1) Is a Tumor Suppressor Protein p53 Target Gene. *Annals New York Academy of Sciences* **878**:638-641 (1999).

69. Sun Y, Zeng X-R, Wenger L, Firestein G & Cheung H. p53 Down-Regulates Matrix Metalloproteinase-1 by Targeting the Communcations Between AP-1 and the Basal Transcription Complex. *Journal of Cellular Biochemistry* **92**:258-269 (2004).

70. Benbow U, Maitra R, Hamilton J & Brinckerhoff C. Selective Modulation of Collagenase 1 Gene Expression by the Chemotherapeutic Agent Doxorubicin. *Clinical Cancer Research* **5**:203-208 (1999).

71. Benbow U, Maitra R, Hamilton J & Brinckerhoff C. Selective Inhibition of Collagenase-1, Gelatinase A and Gelatinase B by Chemotherapeutic Agents. *Annals New York Academy of Sciences* **878**:662-664 (1999).

72. Oikawa T & Yamada T. Molecular biology of the Ets family of transcription factors. *Gene* **303**:11-34 (2003).

73. Wasylyk B, Hahn S & Giovane A. The Ets Family of Transcription Factors. *European Journal of Biochemistry* **211**:7-18 (1993).

74. Sharrocks A. The Ets-Domain Transcription Factor Family. *Nature Reviews. Molecular cell biology* **2**:827-837 (2001).

75. Sharrocks A, Brown A, Ling Y & Yates P. The ETS-domain Transcription Factor Family. *International Journal of Biochemistry and Celluar Biology* **29**:1371-1387 (1997).

76. Sementchenko V & Watson D. Ets target genes: past present and future. *Oncogene* **19**:6533-6548 (2000).

77. Crepieux P, Coll J & Stehelin D. The Ets Family of Proteins: Weak Modulators of Gene Expression in Quest for Transcriptional Partners. *Cricitical Reviews in Oncogenesis* **5**:615-638 (1994).

78. Trojanowska M. Ets factors and regulation of the extracellular matrix. *Oncogene* **19**:6464-6471 (2000).

79. Oikawa T. ETS transcription factors: Possible targets for cancer therapy. *Cancer Science* **95**:626-633 (2004).

80. Sato Y. Role of ETS Family Transcription Factors in Vascular Development and Angiogenesis. *Cell Structure and Function* **26**:19-24 (2001).

81. Karin M, Liu Z-G & Zandi E. AP-1 function and regulation. *Current Opinion in Cell Biology* **9**:240-246 (1997).

82. Kouzarides T & Ziff E. The role of the leucine ziper in the fos-jun interaction. *Nature* **336**:646-651 (1988).

83. Eferl R & Wagner E. AP-1: A Double Edged Sword in Tumorigenesis. *Nature Reviews Cancer* **3**:859-868 (2003).

84. Shaulian E & Karin M. AP-1 in cell proliferation and survival. *Oncogene* **20**:2390-2400 (2001).

85. Shaulian E & Karin M. AP-1 as a regulator of cell life and death. *Nature Cell Biology* **4**:E131-E136 (2004).

86. Angel P & Karin M. The role of Jun, Fos, and the AP-1 complex in cell proliferatoin and transformation. *Biochimica et Biophysica Acta* **1072**:129-157 (1991).

87. Ameyar M, Wisniewska M & Weitzman J. A role for AP-1 in apoptosis: the case for and against. *Biochimie* **85**:747-752 (2003).

88. Birchmeir C, Birchmeier W, Gherardi E & Vande Woude G. Met, Metastasis, Motility and More. *Nature Reviews. Molecular cell biology* **4**:915-925 (2003).

89. Corso S, Comoglio P & Giorgano S. Cancer therapy: can the challenge be MET? *TRENDS in Molecular Medicine* **11**:284-292 (2005).

90. Davis R. The Mitogen-activated Protein Kinase Signal Transduction Pathway. *The Journal of Biological Chemistry* **268**:14553-14556 (1993).

91. Paumelle R, Tulashe DKZ, Plaza S, Leroy C, Reveneau S, Vandenbunder B & Fafeur V. Hepatocyte growth factor/scatter factor activates the ETS1

transcription factor by a RAS-RAF-MEK-ERK signaling pathway. *Oncogene* **21**:2309-2319 (2002).

92. Yang S-H, Sharrocks A & Whitmarsh A. Transcriptional regulation by the MAP kinase signaling cascades. *Gene* **320**:3-21 (2003).

93. Jiang Y, Xu W, Lu J, He F & Yang X. Invasiveness of Hepatocellular Carcinoma Cell Lines: Contribution of Hepatocyte Growth Factor, c-met, and Transcription Factor Ets-1. *Biochemical and Biophysical Research Communications* **286**:1123-1130 (2001).

94. Jinnin M, Ihn H, Mimura Y, Asano Y, Yamane K & Tamaki K. Matrix metalloproteinase-1 up-regulation by hepatocyte growth factor in human dermal fibroblasts via ERK singaling pathway involves Ets1 and Fli1. *Nucleic Acids Research* **33**:3540-3549 (2005).

95. Ozaki I, Mizuta T, Zhao G, Zhang H, Yoshimura T, Kawazoe S, Eguchi Y, Yasutake T, Hisatomi A, Sakai T & Yamamoto K. Induction of multiple matrix metalloproteinase genes in human hepatocellular carcinoma by hepatocyte growth factor via a transcription factor Ets-1. *Hepatology Research* **27**:288-300 (2003).

96. Ozaki I, Zhao G, Mizuta T, Ogawa Y, Hara T, Kajihara S, Hisatomi A, Sakai T & Yamada T. Hepatocyte growth factor induces collagenase (matrix metalloproteinase-1) via the transcription factor Ets-1 in human hepatic stellate cell line. *Journal of Hepatology* **36**:169-178 (2002).

97. Dunsmore S, Rubin J, Kovacs S, Chedid M, Parks W & Welgus H. Mechanisms of Hepatocyte Growth Factor Stimulation of Keratinocyte Metalloproteinase Production. *The Journal of Biological Chemistry* **271**:24576-24582 (1996).

98. Arrieta O, Garcia E, Guevara P, Garcia-Navarrete R, Ondarza R, Rembao D & Sotelo J. Hepatocyte Growth Factor Is Associated with Poor Prognosis of Malignant Gliomas and Is a Predictor for Recurrence of Meningioma. *Cancer* **94**:3210-3218 (2002).

99. Koochekpour S, Jeffers M, Rulong S, Taylor G, Klineberg E, Hudson E, Resau J & Vande Woude G. Met and Hepatocyte Growth Factor/Scatter Factor Expression in Human Gliomas. *Cancer Research* **57**:5391-5398 (1997).

100. Hirose Y, Kojima M, Sagoh M, Murakami H, Yashida K, Shimazaki K & Kawase T. Immunohistochemical examination of c-Met protein expression in astrocytic tumors. *Acta Neuropathology* **95**:345-351 (1998).

101. Brockmann M-A, Ulbricht U, Gruner K, Fillbrandt R, Westphal M & Lamszus K. Glioblastoma and Cerebral Microvascular Endothelial Cell Migration in

Response to Tumor-Associated Growth Factors. *Neurosurgery* **52**:1391-1399 (2003).

102. Abounader R, Ranganathan S, Kim B, Nichols C & Laterra J. Signaling pathways in the induciton of c-met receptor expression by its ligand scatter factor/hepatocyte growth factor in human glioblastoma. *Journal of Neurochemistry* **76**:1497-1508 (2001).

103. Abounader R, Lal B, Luddy C, Koe G, Davidson B, Rosen E & Laterra J. In vivo targeting of SF/HGF and c-met expression via U1snRNA/ribozymes inhibit glioma growth and angiogenesis and promotes apoptosis. *The FASEB Journal* (2001).

104. Andrews N & Faller D. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Research* **19**:2499 (1991).

105. McCawley L & Matrisian L. Matrix metalloproteinases: they're not just for matrix anymore! *Current Opinion in Cell Biology* **13**:534-540 (2001).

106. Somerville R, Oblander S & Apte S. Matrix metalloproteinases: old dogs with new tricks. *Genome Biology* **4**:216 (2003).

107. Fillmore H, Van Meter T & Broaddus W. Membrane type metalloproteinases (MT-MMPs): expression and function during glioma invasion. *Journal of Neuro-Oncology* **53**:187-202 (2001).

108. Van Meter T, Rooprai H, Kibble M, Fillmore H, Broaddus W & Pilkington G. The role of matrix metalloproteinase genes in glioma invasion: co-dependent and interactive proteolysis. *Journal of Neuro-Oncology* **53**:213-235 (2001).

109. Rao J, Steck P, Tofilon P, Boyd D, Ali-Osman F, Stetler-Stevenson W, Liotta L & Sawaya R. Role of plasminogen activator and of 92-Kda type IV collagenase in glioblastoma invasion using an in vitro matrigel model. *Journal of Neuro-Oncology* **18**:129-138 (1994).

110. Rooprai H, Van Meter T, Rucklidge G, Hudson L, Everall I & Pilkington G. Comparative analysis of matrix metalloproteinases by immunocytochemistry, immunohistochemistry and zymography in human primary brain tumors. *International Journal of Oncology* **13**:153-157 (1998).

111. Rutka J, Matsuzawa K, Hubbard S, Fukuyama K, Becker L, Stetler-Stevenson W, Edwards D & Dirks P. Expression of TIMP-1, TIMP-2, 72-and 92-kDa type IV collagenase transcripts in human astrocytoma cell lines: correlation with invasiveness. *International Journal of Oncology* **6**:877-884 (1995).

112. Sawaya R, Yamamoto M, Gokaslan Z, Wang S, Mohanam S, Fuller G, McCutcheon I, Stetler-Stevenson W, Nicolson G & Rao J. Expression and localization of 72 kDa type IV collagenase (MMP-2) in human malignant gliomas in vivo. *Clinical & Experimental Metastasis* **14**:35-42 (1996).

113. Rao J. Molecular Mechanisms of Glioma Invasiveness: The Role of Proteases. *Nature Reviews Cancer* **3**:489-501 (2003).

114. Nabeshima K, Inoue T, Shimao Y & Sameshima T. Matrix metalloproteinases in tumor invasion: Role for cell migration. *Pathology International* **52**:255-264 (2002).

115. Binder D & Berger M. Proteases and the biology of glioma invasion. *Journal of Neuro-Oncology* **56**:149-158 (2002).

116. Ghilardi G, Biondi M, Caputo M, Leviti S, DeMonti M, Guagnellini E & Scorza R. A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-3 Promoter Enhances Breast Cancer Susceptibility. *Clinical Cancer Research* **8**:3820-3823 (2002).

117. Tower G, Coon C & Brinckerhoff C. The 2G single nucleotide polymorphism (SNP) in the MMP-1 promoter contributes to high levels of MMP-1 transcription in MCF-7/ADR breast cancer cells. *Breast Cancer Research and Treatment* **82**:75-82 (2003).

118. Harvey P, Clark I, Jaurand M-C, Warn R & Edwards D. Hepatocyte growth factor/scatter factor enhances the invasion of mesothelioma cell lines and the expression of matrix metalloproteinases. *British Journal of Cancer* **83**:1147-1153 (2000).

119. Kermorgant S, Aparicio T, Dessirier V, Lewin M & Lehy T. Hepatocyte growth factor induces colonic cancer invasiveness via enhanced motility and protease overproduciton. Evidence for PI3 kinase and PKC involvement. *Carcinogenesis* **22**:1035-1042 (2001).

120. Dittmer J. The Biology of the Ets1 Proto-Oncogene. *Molecular Cancer* **2**:29-50 (2003).

121. Karin M. The Regulation of AP-1 Activity by Mitogen-activated Protein Kinases. *The Journal of Biological Chemistry* **270**:16483-16486 (1995).

122. Chinenov Y & Kerppola T. Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* **20**:2438-2452 (2000).

123. Uhm J, Dooley N, Villemure J & Yong V. Mechanisms of Glioma Invasion: Role of Matrix Metalloproteinases. *Canadian Journal of Neurological Sciences* **24**:3-15 (1997).

124. Yordy J & Muise-Helmericks R. Signal transduction and the Ets family of transcription factors. *Oncogene* **19**:6503-6513 (2000).

125. Janknecht R. Analysis of the ERK-Stimulated ETS Transcription Factor ER81. *Molecular and Cellular Biology* **16**:1550-1556 (1996).

126. Bower K, Fritz J & McGuire K. Transcriptional repression of MMP-1 by $p21^{SNFT}$ and reduced in vitro invasiveness of hepatocarcinoma cells. *Oncogene* **23**:8805-8814 (2004).

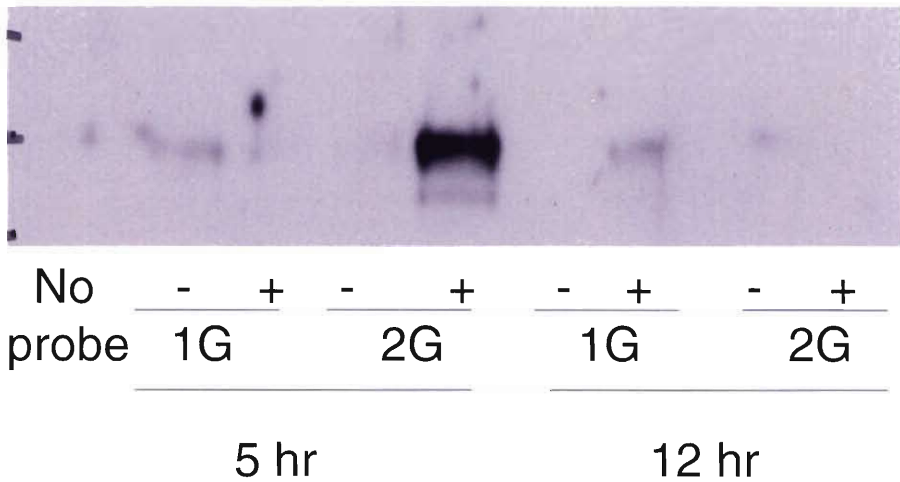
APPENDICES

Appendix I.i DNA-Protein Affinity Pull Down Assay Protocol

This assay determines the ability of proteins to bind to a piece of DNA mimicking the MMP-1 promoter. Before beginning the assay we performed PCR to prepare the double stranded biotin labeled probe. The primer sequences were as follows: forward 5'- biotinCCCTCTTGAACTCACATGTTATG-3'; reverse 5'-GCGTCAAGACTGATATCT-TACTC-3'. The forward primer was aliquoted upon receipt from the company and only thawed one time. This ensured that each PCR began with a fresh aliquot of biotinylated primer. We used 100 ng of genomic DNA isolated from either T98 glioma cells (1G probe) or U251 glioma cells (2G probe). The PCR conditions were as follows: 0.6 µM each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1x PCR Buffer w/o $MgCl_2$, 1 unit Platinum Taq in a total volume of 25 µL. The PCR annealing temperature was 62°C and there were a total of 35 cycles. The PCR product was run on a 2% agarose gel to ensure the expected size piece of 110bp. The product must be purified by ethanol precipitation and resuspended in 60µL of TE pH 8.0. The purified probe was run on an agarose gel to quantify the amount relative to the DNA ladder. Two rounds of PCR were necessary to obtain enough probe for the assay.

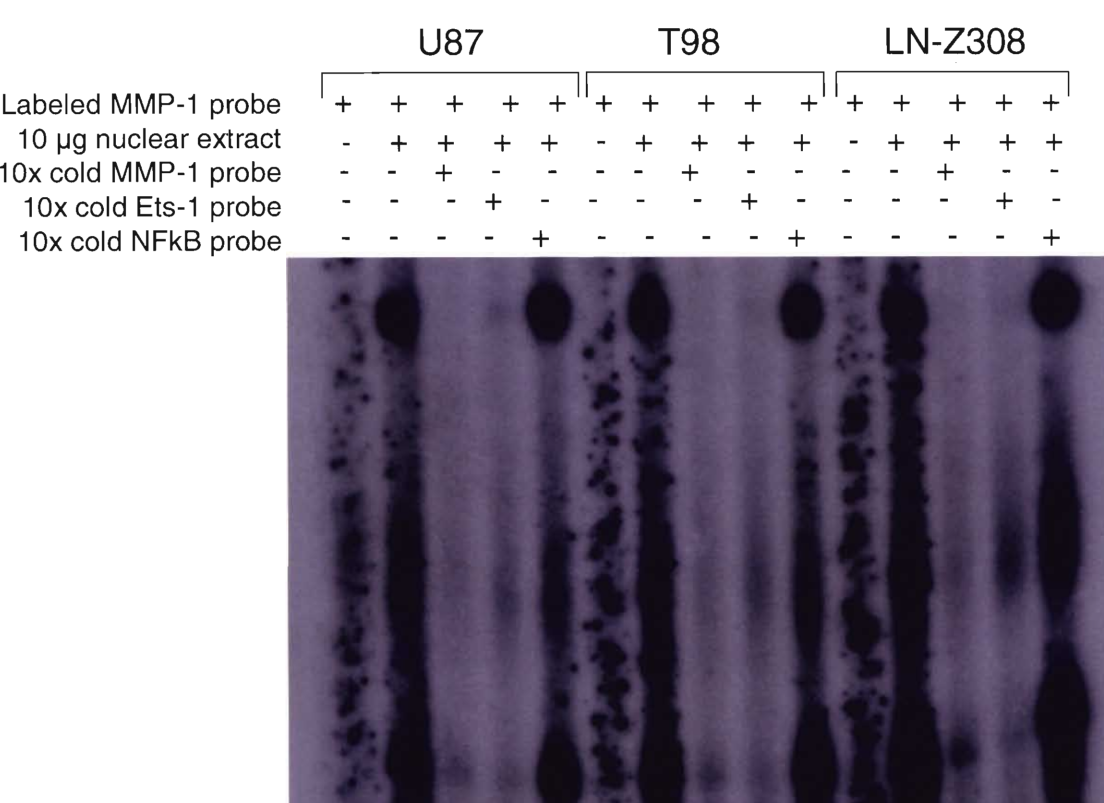
2×10^6 U251 cells were plated in 10 % DMEM. 24 hours post plating the cells were rinsed with PBS and starved from serum overnight. 30ng/mL of HGF was added to 3 mLs of fresh serum free media the following morning. At the desired timepoint the media was removed from the plate, the plates were washed once with PBS and the cells were scraped to the bottom of the plate and transferred to an eppendorf tube with 1.5 mL ice cold PBS. Samples were spun for 10 minutes at maximum speed in a table top centrifuge at 4° C. The cell pellet was either used immediately or frozen at -80°C

for use in the future. Nuclei were extracted following the method described in chapter 2 of this document and nuclear lysates were quantitated with the Coomassie Plus Protein Assay. 60-100 µg of nuclear protein was added to an eppendorf tube to a final volume of 500 µL Lysis Buffer (20 mM HEPES, 10% glycerol, 50mM KCL, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 20 µM $ZnCl_2$, 1 mM DTT, and 0.25% Triton X-100). Protease and phosphatase inhibitors were added just before use. Nuclear extract was precleared with 20 µL Streptavidin-Paramagentic particles (SA-PMP, Pierce) in a rotating wheel at 4˚C. [The SA-PMPs must be washed three times with 1 mL lysis buffer and collected in a magnetic stand 15 minutes prior to use.] Following the pre-clearing step the SA-PMPs were recovered in a magnetic stand. 1µg of the biotinylated oligonucleotide and 4 µg testes DNA were added to the pre-cleared supernatant and allowed to rotate at 4˚C for 30 minutes after which 20 µL of washed SA-PMPs was added and the samples continued rotating for 2 hours at 4˚C. The SA-PMP-DNA complexes were washed three times with 1 mL lysis buffer. After each wash the supernatant was discarded. After the final wash, 20 µL of Laemmli Sample Buffer:BME (950:50) was added to each sample. The samples were boiled for 5 minutes and loaded into a Tris-Gly Gel (Invitrogen). The gel was run at 125 V for two hours and transferred at 25 V for two hours.



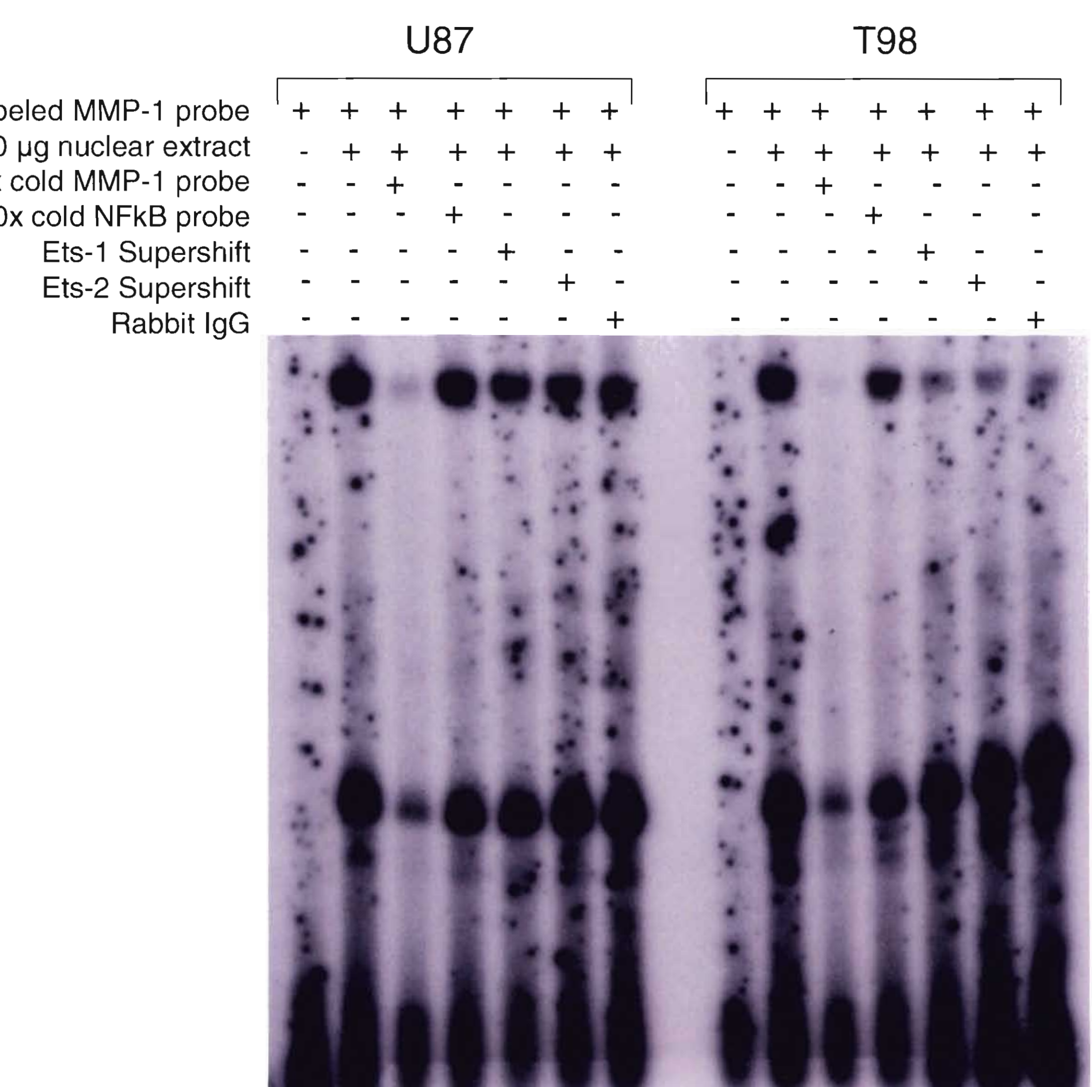
Appendix I.ii DNA-Protein Affinity Pull Down Assay cFos

The AP-1 protein cFos is able to bind to a biotinylated probe mimicking the 2G promoter following HGF/SF treatment for 5 hours. These results are in agreement with the ChIP assay that also indicated that cFos bound to the endogenous 2G promoter at 5 hours. Results from the ChIP assay also suggest that cFos is bound to the 1G promoter. There is a slight band in both the 5 hour and 12 hour lane that may correspond to cFos bound to the 1G promoter.



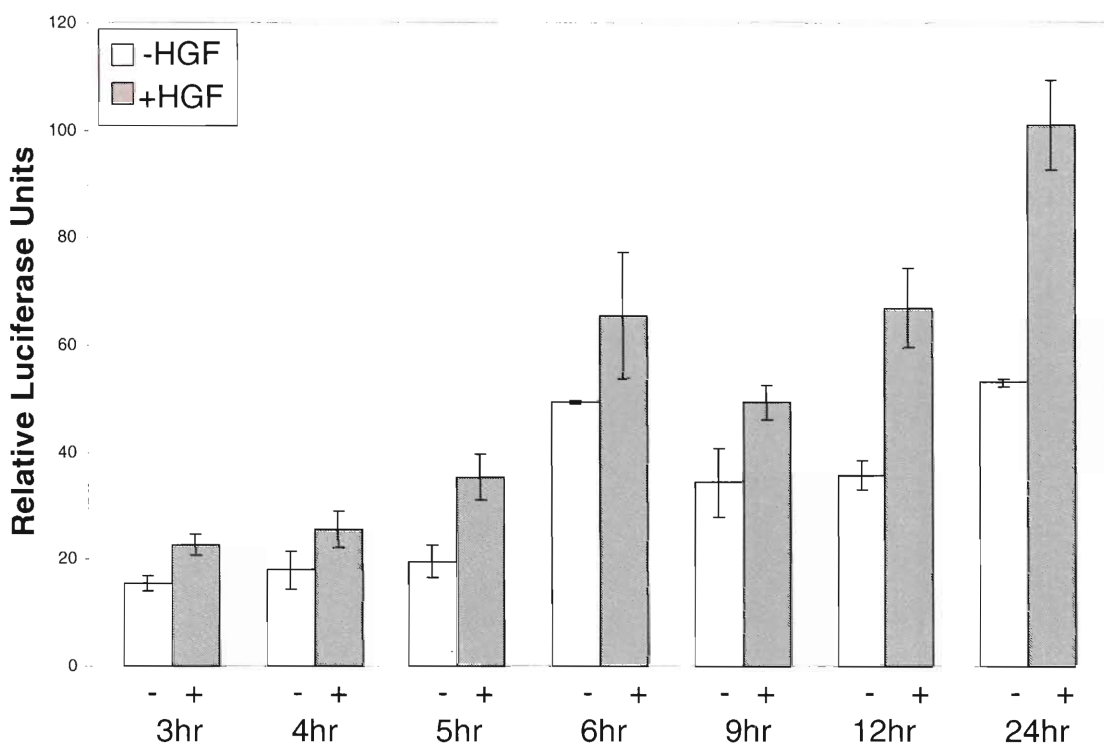
Appendix I.iv Electromobility Shift Assay -- 1G probe

We performed an EMSA with a double stranded radionucleotide labeled probe mimicking the distal 1G MMP-1 promoter to determine if there was a difference in the banding pattern between the 1G and the 2G promoter probes. The data seems to suggest that there are similar proteins bound to both promoters however further experiments would be needed to confirm this initial observation.



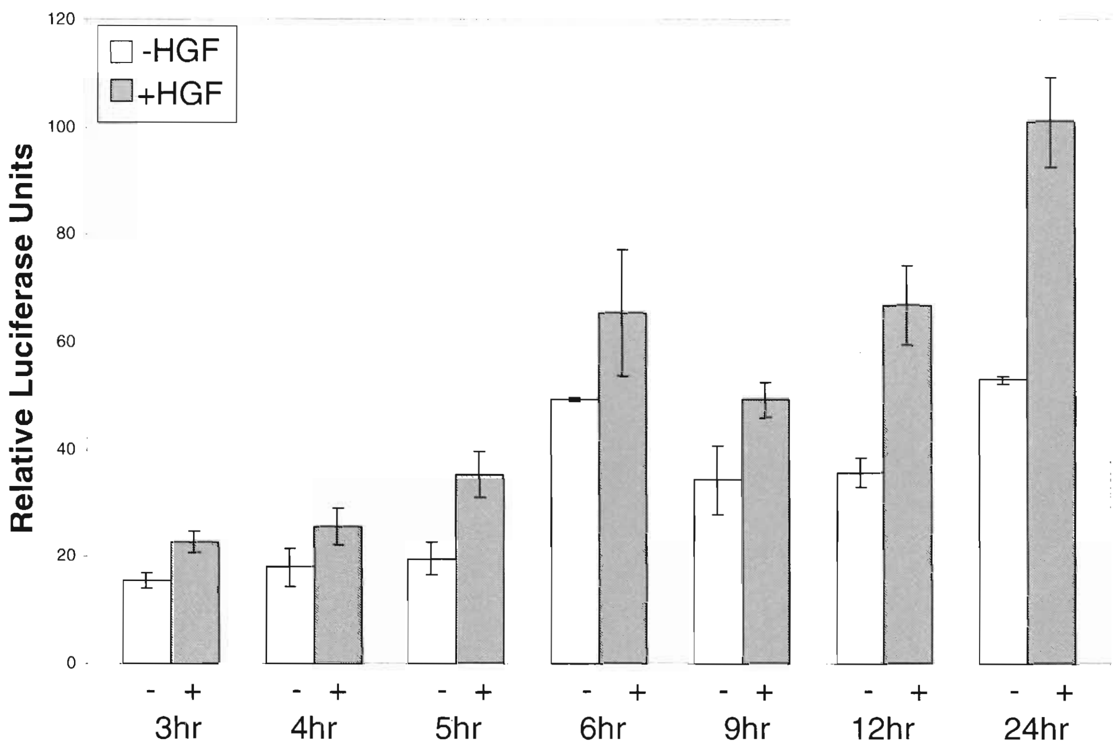
Appendix I.iii Electromobility Shift Assay – Supershift.

We performed an EMSA with antibodies to both Ets-1 and Ets-2 to determine if these are possible proteins bound to a probe mimicking the distal 2G promoter. Neither antibody formed a complex with the DNA-probe complex as indicated by the lack of a supershift of the band shift. This does not rule out these proteins as candidates of proteins bound to the probe because it could be a limitation of the antibody that is interfering with the protein-DNA-probe complex formation.



Appendix II.i Promoter Activation 1G Promoter -/+ HGF/SF

We peformed a luciferase reporter assay with the full length MMP-1 1G promoter construct (obtained from Constance Brinckerhoff, PhD) to determine the response of the 1G promter to HGF/SF treatment in glioma cells. The 1G promoter responds to the HGF/SF beginning at approximately three hours and continues at least for 24 hours. It is not surprising that the 1G promoter responds to HGF/SF since it contains many binding sites that may be responsive to the growth factor including the necessary ETS element located at position -87 in the proximal promoter.



Appendix II.ii Promoter Activation 2G Promoter -/+ HGF/SF

We peformed a luciferase reporter assay with the full length MMP-1 2G promoter construct (obtained from Constance Brinckerhoff, PhD) to determine the response of the 2G promter to HGF/SF treatment in glioma cells. The peak of 2G promoter activity is 12 hours after HGF/SF treatment but the promoter activity begins at three hours in response to the growth factor and continues for at least 24 hours.

VITA

Jessica McCready was born in Miami, Florida and moved to Poughkeepsie, NY at the age of three. She graduated from Our Lady of Lourdes High School in Poughkeepsie NY and completed her undergraduate degree in Nutritional Biochemistry at Cornell University in Ithaca, NY. After graduation she worked for Abbott Laboratories in Waukegan, IL for six months before moving to Richmond, VA to work as a laboratory technician for Dr. Shirley Taylor at Virginia Commonwealth University. She worked there for approximately two years and then began working for Dr. William Broaddus as a laboratory technician. After working for Dr. Broaddus for approximately one year she entered the PhD program in Anatomy and Neurobiology at Virginia Commonwealth University with Dr. Helen Fillmore as her advisor. Jessica studied a single nucleotide polymorphism in gliomas for her graduate project. While in graduate school she was a teacher's assistant in gross anatomy, histology and neuroanatomy. During the course of her study she attended many conferences and gave oral presentations at two international conferences on brain tumor invasion. She has published one paper on her doctoral work in the International Journal of Cancer. Jessica will be working for Dr. Daniel Jay at Tufts University as a post doctoral fellow studying brain tumor invasion after she graduates from Virginia Commonwealth University.