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The Role Of Fra-1 And Cd44 In Malignant Brain Tumor Cell Migration Rasheena Decarroll Edmondson

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The Role of Fra-1 and CD44 in Malignant Brain Tumor Cell Migration

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Biographical Sketch

Rasheena DeCarroll Edmondson was born January 14, 1988, in Wilson, North Carolina to Robert and Judy Edmondson. She was raised in the small town of Lucama, North Carolina. From 2006 to 2010, she attended Elizabeth City State University where she received a Bachelor of Science degree in Biology. She is a candidate for the Master of Science degree in Biology in 2012.

Dedication

To my grandfather, Robert Clanzo Edmondson, and beautiful grandmother, Lillie Bell Forsythe.

I miss and love you both so much. I dedicate this in memory of you.

Acknowledgements

My deepest gratitude goes to God, the giver of wisdom, strength, and all good things. I would like to thank my family for their continued love, support, and belief in me. I would especially like to thank my advisor, Dr. Patrick Martin for his guidance through my research and study at North Carolina A&T University. I would like to acknowledge my committee members, Dr. Perpetua Muganda and Dr. Zandra Pinnix for their support and words of advice. I also wish to acknowledge my fellow colleagues and all members of the Martin Laboratory for filling a lot of my hard days with smiles and laughter. Thank you all.

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List of Symbols and Nomenclature

AER	Apical ectoderm ridge
AIC	5-aminoimadozole-4-carboxamide
AICD	Activation-induced cell death
AP-1	Activator protein 1
BAD	Bcl-2-associated death promoter
BSA	Bovine serum albumin
CD44s	CD44 standard
CD44v	CD44 variant
CNS	Central nervous system
C-terminal	Carboxyl terminal
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGR1	Early growth response 1
eIF4E	Eukaryotic translation initiation factor 4E
ERK	Extracellular signal-regulated kinases
ETS	E-twenty-six
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF-8	Fibroblast growth factor 8

Fra-1	Fos-related antigen 1
GBM	Glioblastoma multiforme
GM-CSF	Granulocyte macrophage colony stimulating factor
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HIF-1α	Hypoxia-inducible factor 1 alpha
ICAM-1	Intercellular adhesion molecule 1
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
JNK	Jun N-terminal kinase
KO	Knock out
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinases
MDR-1	Multiple drug resistant 1
MEK	MAPK/ERK kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MTIC	Methyltriazen-1-yl-imidazole-4-carboxamide
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffered saline
PGP	P-glycoprotein
PI3K	Phosphoinositide-3-kinase

PVDF	Polyvinylidene Fluoride
SF	Scatter factor
shRNA	Small hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
STAT3	Signal transducer and activator of transcription
TMZ	Temozolamide
TPA	12-O tetradecanoylphorbol 13-acetate
Tyr	Tyrosine
uPA	Urokinase-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
wt	Wild type

Abstract

Glioblastoma multiforme (GBM) is the most common form of primary brain tumors. It is a highly aggressive tumor, characterized by an increased proliferation rate and a high capacity to invade surrounding tissues. Currently, GBM treatment includes surgical resection followed by chemotherapy and radiation treatment. However, these therapeutic options often do not alter the infiltrative and migratory capacity of GBMs. The invasive migration of glioblastoma multiforme has been associated with CD44, a cell surface glycoprotein and the principle receptor for hyaluronic acid (HA). The molecular basis of CD44 regulation in GBM is not fully understood. However, CD44 regulation has been shown to be associated with an AP-1 transcription factor family member, fos-related antigen 1 (Fra-1), in an equally aggressive cancer, mesothelioma. Recently, it has also been shown that CD44 expression is linked to Fra-1 expression and activity in glioma cells as well.

To determine whether malignant brain tumor cell migration is mediated by Fra-1 expression, wound healing assays were conducted using the A172 GBM cell line and its clones. Compared to A172 parental cells, which express normal Fra-1 levels, A172-mw6 cells, express a dominant negative version of Fra-1, which decreases the level of Fra-1. The A172-mw6 cell line exhibits a slower migration rate than the A172-mw12 cell line, which contains a vector resulting in increased Fra-1 expression. This confirmed that increased Fra-1 expression promotes CD44-mediated migration of human GBM cells. This study also demonstrated that the MAPK and AKT/mTOR signaling pathways are involved in regulating the expression and activity of Fra-1 as well as the expression of CD44. Inhibition of both pathways decreases phosphorylated Fra-1 expression; however, only inhibition of the AKT pathway decreases total Fra-1 and CD44 expression. Moreover, AKT inhibition significantly decreases glioma cell migration, whereas

MAPK inhibition only slightly decreases the migratory capacity of GBM cells. In conclusion, the results of this study collectively suggest that: (1) Fra-1 and CD44 expression are regulated by the MAPK and/or AKT/mTOR signal transduction pathways in GBM; (2) Fra-1 regulates CD44 expression in human GBM cells, which in turn affects glioma cell migration.

CHAPTER 1

Introduction

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor. Unfortunately, it is also a Grade IV tumor, characterized by the highest proliferation rate and the highest capacity to invade surrounding tissues (Wei et al., 2010). GBM has a yearly incidence rate of ~16.5 per 100,000 persons. The grade IV tumor is accompanied with a poor prognosis and the median survival rate is only 10 to 14 months upon diagnosis (Helseth et al., 2010). Currently, GBM treatment includes surgical resection followed by chemotherapy and radiation treatment. GBM tumors are often resistant or develop resistance to the latter two treatments. The highly invasive nature of glioma cells often prevents the complete removal of the tumor during surgical resection, resulting in a greater chance of recurrence (Helseth et al., 2010). To improve the prognosis of GBM, the disease must be studied at the molecular level in order to better understand its infiltrative and migratory nature.

Cell migration is an essential process that underlies the development and functioning of uni-cellular and multi-cellular organisms. It is a vital part of many normal and pathogenic processes including embryogenesis, the immune response, wound healing, and the spread of cancer (DeLisser, 2009; Kurosaka & Kashina, 2008). Malignant glioma cells are highly invasive tumor cells. There are many underlying factors and mechanisms that regulate the process and allow glioma cells to metastasize and migrate to surrounding healthy tissue in the brain (Soroceanu et al. , 1999). Factors and mechanisms that regulate the process of GBM cell migration include cell-ECM interactions, direct cell-cell contact, growth factors and other microenvironmental factors (Claes et al., 2007). The interaction between adhesion molecules

present on tumor cells with ECM components results in the degradation of the ECM. This allows the cancer cell to migrate and spread to healthy tissue.

The invasive migration of glioblastoma multiforme has been highly associated with the adhesion molecule CD44. CD44 is the principle receptor for hyaluronic acid (HA), a major component of the ECM within the brain. The molecular basis of how CD44 is regulated in GBM is not fully understood. However, CD44 has been shown to be associated with an AP-1 transcription factor, fos-related antigen 1 (Fra-1), in an equally aggressive cancer, mesothelioma (Ramos-Nino et al., 2007; Ramos-Nino et al., 2003). It has been shown that increased expression of Fra-1 in mesothelioma cells correlates with increased levels of CD44 expression (Ramos-Nino et al., 2007). Fra-1 is also involved in regulating the expression of target genes such as *CD44* (Young & Colburn, 2006). Debinski and Gibo have shown that Fra-1 is often overexpressed in glioma cells and that the transcription factor plays a role in the cellular transformation of glioma cells (Debinski & Gibo, 2005). Fra-1 has been shown to regulate the expression of CD44 in glioma cells. It has also been shown that CD44-mediated cell adhesion is promoted by Fra-1 expression and activity (Raines, 2010). Adhesion is necessary for the migration and invasion of tumor cells into healthy surrounding tissue; therefore, we must investigate the role of Fra-1 regulated CD44 expression in the invasive migratory behavior of glioblastoma multiforme.

In this study, we investigated the regulation of CD44 expression by Fra-1. The objective was to determine whether the expression levels of Fra-1 affect CD44-associated cell migration in human glioblastoma multiforme cells. This thesis hypothesizes that decreased expression and activity of Fra-1 correlates with decreased CD44 expression on glioblastoma multiforme, resulting in a decrease in the migratory capacity of GBM cells. To achieve the goals of this study, the three specific aims were: (1) to determine whether the modulation of Fra-1 and CD44

affects GBM cell migration; (2) to determine whether Fra-1 regulates CD44 expression through a MAPK and/or AKT-mediated mechanism; (3) and to determine whether Fra-1 knockdown via pathway inhibition prevents glioma cell migration.

CHAPTER 2

Literature Review

2.1 Glioma

Gliomas are the most common type of primary brain tumors. Glial cells, found in the brain or spinal cord, are the cells which give rise to gliomas (Children's Brain Tumor Foundation [CBTF], 2009). They are known as the supportive cells of the central nervous system (CNS) that surround neurons, providing insulation, energy, and nutrients. There are three types of glial cells: astrocytes, microglia, and oligodendrocytes (Johns Hopkins Medicine, 2011; Yokoyama et al., 2004). Astrocytes are star-shaped glial cells found in the brain's capillaries that form the blood-brain barrier (Johns Hopkins Medicine, 2011). They provide neurons with physical support by forming a matrix to keep them in place. Astrocytes also provide nourishment to neurons by receiving glucose from capillaries and processing the glucose so that it can be used for energy (Yokoyama et al., 2004). Another function of the star-shaped glial cells is to remove neural debris such as dead neurons via phagocytosis. Microglia, the smallest types of glial cells, are responsible for "housekeeping" in the central nervous system (Yokoyama et al., 2004). Microglia constantly move about the central nervous system, recognizing and destroying damaged or infected neurons, bacteria, viruses, and other infectious agents. These cells destroy organisms that have invaded the brain by phagocytosis and by releasing cytotoxic substances such as nitric oxide and hydrogen peroxide that can directly destroy damaged nerve cells. Microglia can also produce cytokines such as Interluekin-1 (IL-1) and the tumor necrosis factor (TNF), which promote the demyelination of axons of unhealthy neurons (Yokoyama et al., 2004). Oligodendrocytes produce a fatty protein called myelin, which surrounds the axons of nerve cells, forming the myelin sheath. The myelin sheath provides the axons with insulation

(Yokoyama et al., 2004). Brain tumors are diagnosed and then named based on a classification system. Over the years, there have been several grading systems used to classify tumors of the central nervous system including the Kernohan's classification system, the Ringertz classification system, the St. Anne/Mayo system, and the World Health Organization (WHO) grading scheme. The most widely used brain tumor classification system is the WHO grading system. The World Health Organization grades tumors on a scale from I to IV, from least to most malignant, based on their microscopic appearance using the following criteria: atypia (similarity to normal cells), mitotic index (growth rate), vascularity (blood supply), necrosis (presence of dead cells in the center of the tumor), and its potential to invade or spread to adjacent tissues (Johns Hopkins Medicine, 2011). A table describing the WHO tumor grading system is available in the appendix.

Grade I tumors are the least malignant, grow slowly, and appear to be almost normal under the microscope. Grade I tumors are usually cured with surgery alone, and are often associated with long-term survival (American Association of Neurological Surgeons [AANS], 2006; Johns Hopkins Medicine, 2011). Grade II tumors grow slightly faster than Grade I tumors and appear slightly abnormal under the microscope. Tumors classified as Grade II may spread into normal surrounding tissue, and can recur as a higher grade tumor.

Grade III tumors are malignant and possess actively reproducing abnormal cells (AANS, 2006). These tumors appear abnormal under the microscope and have the ability to invade surrounding normal brain tissue. Grade III tumors recur frequently, often at a higher grade. Grade IV tumors are the most malignant, and have cells that appear highly abnormal under the microscope, and reproduce quickly. Grade IV tumors invade wide areas of adjacent normal brain tissue, and form new blood vessels in order to maintain its rapid growth (AANS, 2006;

Johns Hopkins Medicine, 2011). Glioblastoma multiforme (GBM), the most common, aggressive, and proliferative brain tumor, is a Grade IV tumor. Tumors in the central nervous system often contain several grades of cells. The highest grade of cells determines the overall grade of the tumor, even if the majority of the tumor is of a lower grade.

2.2 Glioblastoma Multiforme (GBM) and Current Treatment

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor. Unfortunately, it is also a Grade IV tumor, the most malignant type, characterized by the highest proliferation rate and highest capacity to invade surrounding normal tissue (Wei et al., 2010). Glioblastoma multiforme typically affects adults at 45 years of age and above, and has a yearly incidence rate of ~16.5 per 100,000 persons. The median survival rate for individuals with GBM is 10 to 14 months (Helseth et al., 2010). The recommended treatment of Glioblastoma multiforme is surgical tumor resection followed by chemotherapy and radiation treatment. GBM tumors are often resistant to or develop resistance against radiotherapy and chemotherapy. The highly invasive nature of glioma cells often prevents the complete removal of the tumor during surgical resection resulting in a great chance of recurrence (Helseth et al., 2010). Therefore, despite the aggressive multimodal therapy used to treat Glioblastoma multiforme, the disease remains incurable. GBM has one of the worst 5-year survival rates among all human cancers (Krex et al., 2007). Within two years from the time of primary surgery, almost all GBM patients will experience tumor regrowth and eventually succumb to the aggressive disease. However, a small percentage (<3%) of individuals diagnosed with GBM actually survive for more than 36 months. These patients are referred to as long-term survivors of Glioblastoma multiforme (Krex, et al., 2007; Xu et al., 2010).

There are several medications used to treat or reduce the symptoms of Glioblastoma multiforme including the two popular drugs of choice, temozolamide (TMZ) and bevacizumab. Temozolamide is a cytotoxic alkylating agent that, when hydrolyzed, inhibits the replication of DNA by the methylation of the nucleic acid (Darkes et al. , 2002; Friedman et al., 2000). TMZ is a small lipophilic molecule with a molecular weight of 194 daltons that is readily absorbed in the digestion tract, and able to cross the blood-brain barrier (Agarwala & Kirkwood, 2000). Once the drug reaches an environment with a pH above 7, such as the blood and tissues, TMZ spontaneously hydrolyzes to the active metabolite methyltriazen-1-yl imidazole-4-carboxamide (MTIC) (Agarwala & Kirkwood, 2000; Darkes et al., 2002). MTIC then breaks down rapidly to an inactive carboxylic acid derivative, 5-aminoimadazole-4-carboxamide (AIC), and a highly reactive methyldiazonium ion (Darkes et al., 2002). The methyldiazonium ion methylates the guanine bases of the DNA molecule, forming O^6-methylguanine and N^7-methylguanine. It is the formation of O^6-methylguanine that is responsible for the cytotoxic effects of the chemotherapy agent. When enzymes responsible for correcting DNA mismatches attempt to remove O^6-methylguanine, they make single-stranded and double-stranded breaks in the DNA molecule, causing apoptotic pathways to become active (Agarwala & Kirkwood, 2000). Although the initial effects of temozolamide are satisfactory, GBM often becomes resistant to the drug over time. There are several cellular mechanisms of resistance for TMZ including defects in DNA mismatch repair, increased repair of breaks in the DNA strand, and increased removal of alkyl groups by specific enzymes (Agarwala & Kirkwood, 2000).

Bevacizumab is a humanized monoclonal antibody that targets the protein vascular endothelial growth factor (VEGF) (Bergsland & Dickler, 2004; Chamberlain, 2011). Many cancerous cells, including GBM cells, often overexpress VEGF, which stimulates angiogenesis.

In part, the degree of VEGF expression in Glioblastoma multiforme correlates with the grade and the aggressiveness of the tumor, as well as the clinical outcome (Chamberlain, 2011). By inhibiting VEGF expression, bevacizumab helps to prevent blood vessels from reaching the tumor, leading to tumor starvation and shrinkage. However, prolonged treatment with the anti-angiogenic therapy leads to increased migration and invasion (de Groot et al., 2010). Bevacizumab leads to major blood vessel remodeling, which results in reduced perfusion and increased tumor hypoxia (Keunen et al., 2011). As a result, tumor glycolysis occurs, which leads to an increase in lactic acid production and to the stabilization of hypoxia-inducible factor-1 alpha (HIF-1 alpha). The hypoxia-inducible factor-1 alpha upregulates the expression of genes related to cancer aggressiveness such as the E-twenty-six (ETS) and matrix metalloproteinase (MMP) family, leading to increased invasion into surrounding normal brain tissue (Keunen et al., 2011).

Temozolomide was shown to decrease proliferation in two glioblastoma cell lines; however, the migrational behavior of the glioma cells was unaffected by the drug (Gunther et al., 2003). It has also not been shown for bevacuzimab treatment to decrease cancer cell migration. In fact, chronic exposure of colorectal cancer cells to bevacuzimab has been shown to enhance cellular migration and invasion (Fan et al., 2011). Indeed, TMZ and bevacizumab, as well as other medications, are successful at decreasing or inhibiting the growth of brain tumors. However, these effects are usually only temporary, due to the tumor cell's ability to develop resistance against the drug or to the drug affecting other pathways, causing adverse side effects. It is essential to do more research in order to better understand the mechanisms that govern the invasive and migratory nature of Glioblastoma multiforme so that we may develop drugs to

target a specific pathway without the tumor developing resistance or having adverse effects via another pathway.

2.3 Invasion and Migration of GBM

Cell migration is an essential process that underlies the development and functioning of uni-cellular and multi-cellular organisms. It is a vital part of many normal and pathogenic processes including embryonic development, the immune response, wound healing, and the spread of cancer (Kurosaka & Kashina, 2008). It is crucial for cell migration to be correctly coordinated during embryogenesis. Defects in migration may result in severe embryonic malformations ranging from birth defects to early embryonic lethality (Kurosaka & Kashina, 2008). Cells of the immune system are recruited and migrate to the site of infection in order to rid the body of invading pathogens. If an individual is cut, cells of the immune system including neutrophils, macrophages, and fibroblasts migrate to the wound and participate in wound healing. Cell migration also plays a role in the spread of cancer. In metastasis, the cancerous cells migrate from the initial site of tumor growth as single cells or in small groups to healthy surrounding tissue.

Malignant glioma cells are highly invasive tumor cells. There are many underlying factors and mechanisms that regulate the process allowing glioma cells to spread and migrate to adjacent healthy brain tissue (Soroceanu et al., 1999). It is believed that highly invasive GBM cells temporarily go into cell cycle arrest during migratory phases, and therefore become resistant to the majority of chemotherapy and radiotherapy treatments that are currently available (Soroceanu et al., 1999). Once the migrating cells have traveled a certain distance from the primary tumor, they reenter the cell cycle and form recurrent tumors. Glioblastoma multiforme cell migration is a multistep process that requires a number of molecular interactions contributed

to by the malignant glioma cell, the surrounding extracellular matrix (ECM), and stromal cells (Geho et al., 2005). Factors and mechanisms that regulate the process of GBM cell migration include glioma cell-ECM interactions, direct cell-cell contact, growth factors and other microenvironmental factors (Claes et al., 2007; Geho et al., 2005).

The migration of glioma cells requires the expression of adhesion molecules, adequate positioning of these molecules, attachment to its appropriate substrate, and detachment when the cell moves on (Claes et al., 2007). CD44 and integrins are major adhesion molecules present on glioma cells. Integrins are heterodimeric structures, composed of an α and β subunit, which extend across the cellular membrane (Domínguez-Giménez et al., 2007) . The extracellular domain of integrins recognizes and binds to specific proteins of the extracellular matrix (Domínguez-Giménez et al., 2007; Geho et al., 2005). The intracellular domain associates with specific cytoskeletal adaptor proteins. Therefore, integrins play an important role in cell adhesion to the ECM as well as in the transmission of signals from the ECM to the inside of the cell, which influences multiple cellular activities including migration (Domínguez-Giménez et al., 2007). Once glioma cells are attached to the ECM, in order to metastasize, the cancer cell must degrade the extracellular matrix (Geho et al., 2005). GBM cells produce proteolytic enzymes such as matrix metalloproteinases (MMPs) that degrade ECM components. Once the tumor cell degrades the ECM, it is able to migrate, spreading to healthy tissue (Claes et al., 2007). CD44 is another adhesion molecule that is highly expressed by glioma cells. CD44 is the main cell surface receptor for hyaluronic acid (HA). Increased expression of CD44 correlates with a higher glioma grade. Studies have shown that increased expression of the HA receptor correlates with increased GBM invasion and migration (Claes et al., 2007).

2.4 The Adhesion Molecule CD44

CD44 is a family of transmembrane glycoproteins encoded by a highly conserved gene that contains 20 exons (Merzak et al., 1994). The CD44 proteins are involved in cell adhesion, cell-cell communication, and signal transduction. Alternative splicing of CD44 mRNA and posttranslational modifications result in a multitude of CD44 isoforms, which correlates with a variety of different functions. Up to 10 variable exons can be inserted into the extracellular domain during splicing. The CD44 gene has been mapped to the chromosomal locus 11p13. The gene is made up of two groups of exons. Exons 1-5 and 16-20 make up one group, and are spliced together to form CD44s, the most common isoform. The other group, exons 6-15, are known as the variable exons, which can be inserted between exons 5 and 16, forming the variant CD44 isoforms (Goodison et al., 1999). The variant exons 6-15 may also be referred to as v1-10 (Bajorath, 2000; Goodison et al., 1999). The molecular mass of the standard isoform, CD44s, is approximately 37 kDa; however, the molecular weight is often increased to ~80kDa as a result of glycosaminoglycan side chain attachment during posttranslational modification (Goodison et al., 1999; Sneath & Mangham, 1998). The variant isoform that contains all variant exons (v1-10) may have a molecular weight of up to 200 kDa (Goodison et al., 1999).

The CD44 protein consists of three regions: a highly conserved C-terminal cytoplasmic domain, a hydrophobic transmembrane domain, and an extracellular domain which can be further divided into conserved and nonconserved regions. The principle ligand of CD44, hyaluronic acid, as well as other ligands, bind to the extracellular domain of the protein (Figure 2.1) (Goodison et al., 1999).

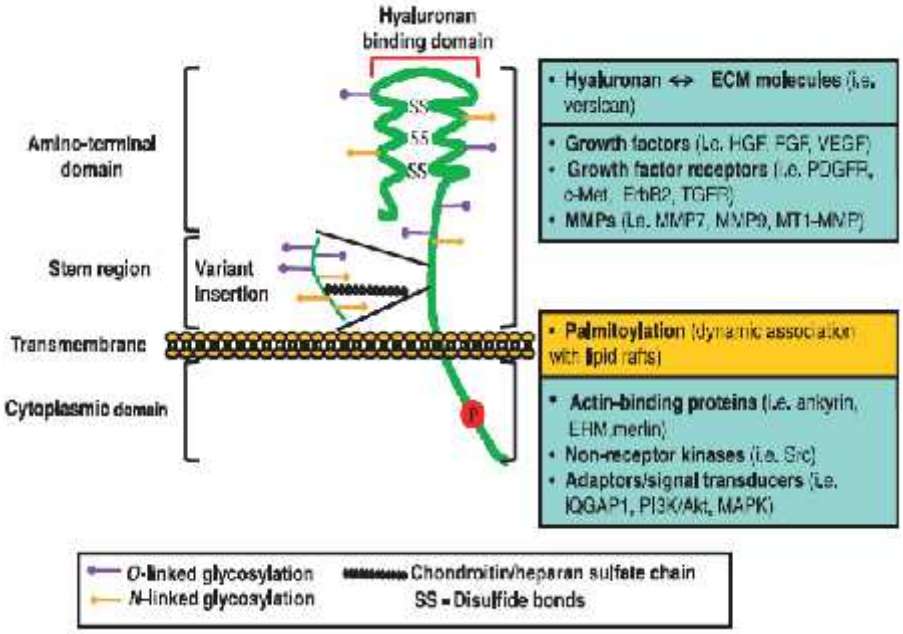

Figure 2.1. Structure, binding domains, and interactions of CD44.

There have been several ligands for CD44 that have been identified including chondroitin sulfate, collagen, fibronectin, osteopontin, growth factors, MMPs, and the principle ligand, hyaluronic acid (Bajorath, 2000). Hyaluronic acid is a linear, polymeric glycosaminoglycan and a major component of the ECM (Bajorath, 2000; Goodison et al., 1999). As shown in Figure 2.2, HA is comprised of repeating D-glucuronic acid and N-acetyl-D-glucosamine dissacharide units (Bajorath, 2000). The CD44 protein has at least three HA binding sites, and exists in three states with respect to binding HA (Misra et al., 2011). The cell surface receptor may be in a non-binding state, a non-binding state unless activated by stimuli, or in a constitutively-binding state. Hyaluronic acid induces signaling within the cell once it binds to CD44 that is constitutively active (Misra et al., 2011). The binding capacity of CD44 to hyaluronic acid can be regulated in a number of ways. It may be affected by glycosylation and the attachment of glycosaminoglycan side chains during posttranslational modifications (Goodison et al., 1999). CD44-HA binding can also be affected by mutations in all regions of the CD44 molecule,

especially the extracellular domain (Bajorath, 2000). Many cancer cell types express CD44 variants, which often have a higher affinity to bind to hyaluronic acid (Sneath & Mangham, 1998). The enhanced ability to bind to HA leads to increased tumorigenecity. All glioma cells have been shown to express the standard isoform of CD44, which lacks all variable exons. However, the expression of CD44 splice variants has been reported in one GBM sample (Frank et al., 1996; Knüpfer et al., 1999).

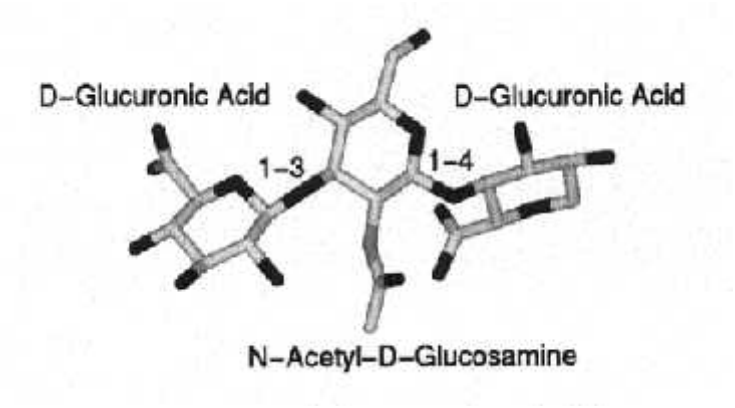

Figure 2.2. Hyaluronic acid structure segment.

2.5 The Normal Function of CD44

The binding of hyaluronic acid to CD44 initiates, or at least influences, most activities of the adhesion molecule (Zöller, 2011). The major function of CD44 is to maintain organ and tissue structure via cell-cell and cell-ECM adhesion; however, due to the variant isoforms of CD44, the protein is also involved in other cellular functions including cellular migration, lymphocyte activation and homing, the presentation of chemokines and growth factors to migrating cells, as well as in the transmission of growth signals via tyrosine kinases (Bajorath, 2000; Goodison et al., 1999; Naor et al., 2008; Sneath & Mangham, 1998). CD44 is also involved in the degradation of hyaluronic acid, and in the transmission of signals mediating hematopoiesis and apoptosis (Naor et al., 2008).

The cell surface protein, CD44, plays an important role in cellular migration. HA binding to CD44 induces changes in the protein's membrane localization and conformation, which triggers the association and activation of various signal transduction molecules and proteases (Zöller, 2011). In order for cells to migrate, they must have the ability to overcome the physical resistance of three-dimensional tissue networks. Proteases, such as the matrix metalloproteases (MMPs) family, participate by aiding in the degradation of several extracellular components including collagen, fibronectin, and laminins (Friedl & Wolf, 2003; Gilles et al., 2001). The degradation of ECM components facilitates the migration of normal and tumor cells to surrounding tissue.

CD44 also plays a crucial role in the body's immune response, particularly in the process of inflammation and wound healing (Puré & Cuff, 2001). CD44 is constitutively expressed on leukocytes and parenchymal cells including endothelial, epithelial, and smooth muscle cells (Puré & Cuff, 2001). Once leukocytes such as T lymphocytes or macrophages have encountered an antigen, cell surface CD44 expression is transiently upregulated (Goodison et al., 1999; Zöller, 2011). The CD44 on T cells may be rapidly activated by cytokines and chemokines that are produced locally at the sites of inflammation as well (Puré & Cuff, 2001). The binding of CD44 on activated T lymphocytes to HA expressed on vascular endothelial cells results in the activation of T Cell extravasation into sites of inflammation (Goodison et al., 1999; Siegelman et al., 2000). CD44-HA interactions contribute to the recruitment of white blood cells from the bloodstream to the site of inflammation, and initially involves the rolling of activated leukocytes along the endothelium (Puré & Cuff, 2001). This leads to the formation of additional adhesive interactions between leukocytes and endothelial cells that are necessary for firmer binding (Johnson-Leger et al., 2000; Puré & Cuff, 2001). Once leukocytes exit the bloodstream and

migrate across the endothelium, they move into the sites of tissue damage or infection (Goodison et al., 1999; Johnson-Leger et al., 2000; Zöller, 2011).

In addition to CD44's ability to mediate cell adhesion and migration, it also acts as an activation molecule, and can promote inflammation through the activation of genes involved in inflammation (Puré & Cuff, 2001). Studies have shown that the binding of HA to CD44 increases vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression on synoviocytes and epithelial cells (Burns et al., 2001; Puré & Cuff, 2001). The interaction of vascular adhesion molecules such as VCAM-1, ICAM-1, and selectins with ligands present on the surface of leukocytes (i.e. integrins) regulate diapedesis, which is the migration of blood cells through intact vessel walls into the surrounding tissue (Foster, 1996).

Through the use of knockout (KO) mice, it has been shown that CD44 plays an important role in activation-induced cell death (AICD) as well (McKallip et al., 2002). AICD is the process in which cells undergo apoptosis in a controlled manner when the cell's receptor interacts with a death factor such as the TNF (Maher et al. , 2002). CD44 knockout mice were treated with the anti-CD3 monoclonal antibody (mAb), which has been shown to induce T cell apoptosis. The treatment of CD44 KO splenocytes with anti-CD3 mAb led to a significantly reduced level of apoptosis compared to CD44 wild type splenocytes (McKallip et al., 2002). CD44 expression has also been shown to promote resistance to apoptosis in colon cancer cells (Lakshman et al., 2004). Although the findings of the studies are contradictory, showing both pro- and anti-apoptotic effects of CD44, they still highlight the fact that the cell surface receptor plays a major role in apoptosis.

CD44 has also been implicated in embryogenesis (DeLisser, 2009; Ponta & Herrlich, 1998; Sherman et al., 1998). CD44 splice variants, including CD44v3 and CD44v6, play a

crucial role in limb development by helping to transport a proliferative signal from cells of the apical ectoderm ridge (AER) to the underlying limb mesenchymal cells. Interactions between the AER and limb mesenchyme are necessary in order to establish limb outgrowth and differentiation (Figure 2.3). An AER cell secretes fibroblast growth factor-8 (FGF-8). The secreted growth factor binds to a CD44 variant protein on the same cell (Sherman et al., 1998). FGF-8 then binds with high affinity to the FGF receptor present on mesenchymal cells, which leads to cell proliferation, and subsequent limb development (Sherman et al., 1998).

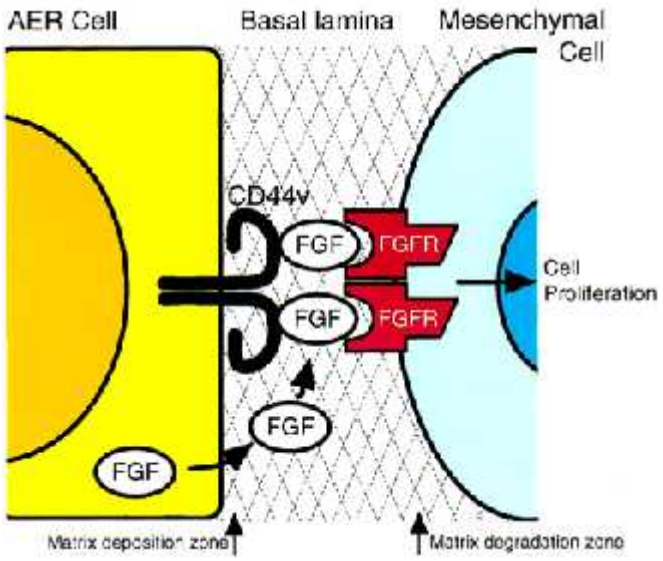

Figure 2.3. CD44-FGF8 interaction in limb development.

2.6 The Oncogenic Function of CD44

A large number of studies have shown a close correlation between malignancy and CD44 expression, as well as with ECM environments rich in hyaluronic acid. When hyaluronic acid binds to the CD44 adhesion molecules present on the tumor cell, tyrosine phosphorylation is either stimulated or inhibited. The CD44 of tumor cells interacts with HA-rich microenvironments, which affects cell signaling pathways that promote the ability of cancer cells

to migrate, to invade the ECM, and spread to healthy tissue (Misra et al., 2011). Cancerous cells often exhibit higher levels of CD44 expression as seen in numerous types of human cancer including breast, colon, prostate, bladder, ovarian, lung, and brain cancer (Yoshida et al., 2012) . Many tumor cells express CD44 splice variants that possess a higher binding affinity for HA. Hyaluronic acid is especially abundant in the white matter tracts of the brain, which are one of the most common routes of GBM invasion (Wiranowska et al., 2010). Since the extracellular matrix of the brain has an abundance of CD44's principle ligand, HA, it provides the ideal environment for the glioma cells to migrate and develop new blood vessels (Wiranowska et al., 2010). The high amounts of HA in the brain and the overexpression of CD44 by glioma cells are therefore greatly involved in the migration, invasion, and metastasis of Glioblastoma multiforme.

CD44 promotes the invasive behavior of cancer through a number of signaling networks. During crosstalk, the CD44 protein interacts with growth factor receptors such as the epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (HGFR). The epidermal growth factor (EGF), the principal ligand of EGFR (Her1), binds to the extracellular domain of the receptor, inducing receptor dimerization. EGFR forms homodimers or heterodimers with other members of the epidermal growth factor family (Jorissen et al., 2003; Schlessinger, 2002). Tyrosine autophosphorylation occurs, triggering a network of signaling processes that promote tumor cell proliferation, migration, adhesion, and angiogenesis, and decrease apoptosis (Arteaga, 2003). A mature hepatocyte growth factor (HGF, also known as scatter factor, SF) protein binds to the extracellular domain of the HGFR, which is also known as c-Met (You & McDonald, 2008). Upon ligand binding, c-Met is activated by the autophosphorylation of two tyrosine residues, Tyr 1234 and Tyr 1235, located in the tyrosine kinase domain (Eder et al., 2009).

Subsequently, two other tyrosines, Tyr 1349 and Tyr 1356 are phosphorylated (You & McDonald, 2008). The phosphorylation of the latter two tyrosine residues leads to the activation of multiple signal transduction pathways that contribute to tumor survival, growth, and invasion. Pathways activated by EGFR stimulation include the Src/focal adhesion kinase (FAK) pathway, the signal transducer and activator of transcription (STAT3) pathway, the mitogen activated protein kinase (MAPK) pathway, and the phosphoinositide-3-kinase (PI3K)/ AKT pathway. In many tumor cells, c-Met expression is activated by HGF through an autocrine loop (You & McDonald, 2008). In other words, the cancerous cells continuously produce and secrete HGF, which bind to the HGF receptor, leading to constitutive activity of many signal transduction pathways.

Activation of the PI3K pathway plays a vital role in many cellular functions contributing to the spread of cancer including cell survival, growth, proliferation, and migration (Hennessy et al., 2005). AKT signaling promotes cell survival by inactivating several pro-apoptotic factors including the Bcl-2-associated death (BAD) promoter, procaspase-9, and the Forkhead (FKHR) family of transcription factors (Hennessy et al., 2005). AKT also promotes cell survival by activating transcription factors that upregulate anti-apoptotic genes (Zöller, 2011). AKT-mediated activation of the mTOR pathway also facilitates cell survival and proliferation (Hennessy et al., 2005). The binding of CD44 to HA and other ligands also stimulates activation of the MAPK/ERK pathway, which also has a well-defined role in cancer biology. The intracellular signaling pathway is involved in a number of cellular functions including cell survival, proliferation, cell cycle regulation, angiogenesis, and migration.

In the same manner that immune cells must extravasate to sites of tissue damage or infection, metastasizing tumor cells must do the same. In many tumor cells, the upregulated

expression of CD44 strengthens their binding to hyaluronic acid present on surrounding endothelial cells, which allows the tumor cells to metastasize and reach healthy tissue (Yoshida et al., 2012; Zöller, 2011). Angiogenesis is essential for invasive tumor growth and metastasis. When CD44 binds to HA, tumors cell produce and secrete proteolytic enzymes that aid in the degradation of the ECM (Misra et al., 2011). The binding of CD44 to HA and other ligands increases the production of growth factors (i.e. VEGF and EGF) by tumor cells. The growth factors then recruit surrounding blood vessels and stimulate them to form new vessels in the tumor. The new vasculature supplies the tumor with oxygen and nutrients, which promotes further growth (Hoeben et al., 2004).

The prognosis for patients with glioblastoma multiforme remains poor, which is largely due to the ability of GBM cells to develop resistance against current drugs (Helseth et al., 2010; Yoshida et al., 2012). The CD44 protein has been shown to contribute to drug resistance in cancer (Cain et al., 2011; Zöller, 2011). CD44 activated by the binding of HA associates with the multiple drug resistant 1 (MDR-1) protein (Zöller, 2011). Increased expression of CD44 correlates with an increased expression of the *MDR1* gene, which encodes a P-glycoprotein (PGP) (Santoni-Rugiu & Silverman, 1997; Zöller, 2011). PGP is an integral membrane protein which functions in drug efflux (Santoni-Rugiu & Silverman, 1997). MDR-1 is expressed in both normal and tumor cells. In normal cells, PGP functions to rid the cell of cytotoxic drugs that are harmful. In tumor cells, the protein encoded by MDR-1 transports chemotherapy drugs out of the cell, therefore inhibiting their anti-cancer effects (Santoni-Rugiu & Silverman, 1997; Zöller, 2011). CD44 also plays a role in drug resistance via the attenuation of the Hippo, JUN N-terminal kinase (JNK), and p53 pathways. The weakening of these apoptotic pathways leads to a decrease in the death of abnormal tumor cells (Zöller, 2011).

Numerous studies have shown that the CD44 adhesion molecule plays a crucial role in the invasive and migratory nature of Glioblastoma multiforme (Merzak et al., 1994; Wei et al., 2010; Wiranowska et al., 2010; You & McDonald, 2008). However, more research must be done in order to determine how CD44 expression is regulated by the AP-1 transcription factor, Fra-1, in GBM. CD44 regulation was first shown to be associated with the AP-1 transcription factor in an equally aggressive cancer, mesothelioma, which affects the mesothelium that covers internal organs such as the heart and lungs (Ramos-Nino et al., 2007; Ramos-Nino et al., 2003). Ramos-Nino et al. showed that by silencing Fra-1 via shRNA and siRNA, the expression of CD44 is decreased in mesothelioma cells (Ramos-Nino et al., 2003). It was later shown that Fra-1 regulates CD44 expression in gliomas via siRNA directed against Fra-1 (Raines, 2010). It is believed that increased expression of the transcription factor leads to the activation of target genes that promote the invasion of cancer cells (Ramos-Nino et al., 2007).

2.7 The Normal Function of Fos-related Antigen 1 (Fra-1)

Fos-related antigen 1 (Fra-1) is a member of the activator protein 1 (AP-1) family of transcription factors, which is involved in a number of cellular processes including cell proliferation, differentiation, and programmed cell death (Chiappetta et al., 2007; Chiappetta et al., 2000; Young & Colburn, 2006). The transcription factor AP-1 is also involved in regulating the expression of target genes (Young & Colburn, 2006). The AP-1 transcription factor family are dimers consisting of Jun-Jun homodimers or Jun-Fos heterodimers (Figure 2.4) (Young & Colburn, 2006). Proteins of the Jun family include c-Jun, JunB, and JunD. Members of the Fos family include c-Fos, FosB, Fra-1, and Fra-2. AP-1 transcription factors are activated by growth factors, hormones, cytokines, ultraviolet radiation, and tumor promoters such as the phorbol ester 12-O tetradecanoylphorbol 13-acetate (TPA) (Bamberger et al., 1999; Young & Colburn, 2006).

The mitogen-activated AP-1 dimer binds to specific DNA sequences in the promoter regions of target genes, several of which are involved in processes such as cellular proliferation and tumor invasion (Bamberger et al., 1999). Studies have shown that Jun-Jun homodimers have little or no DNA-binding activity depending on the target DNA sequences. Jun-Fos heterodimers are more stable and have a higher binding affinity for the promoter regions of target genes than Jun-Jun homodimers (Bamberger et al., 1999). The binding of transcription factors to the promoter region of target genes is necessary for transactivation; therefore, the activity of AP-1 family members is essential for the activation and regulation of downstream genes.

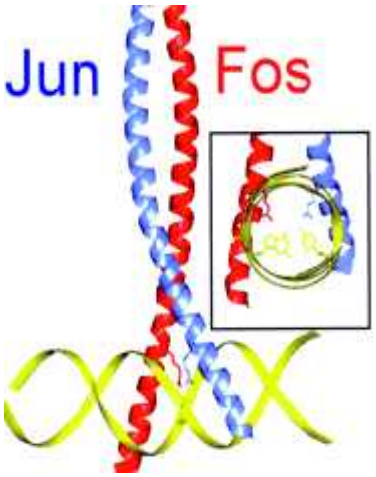

Figure 2.4. Jun-Fos heterodimer.

The fos-related antigen 1 protein plays a role in the central nervous system, but little is actually understood about its actual function. Fra-1 activity is observed in the rat brain during normal development and following brain injuries in adulthood (Pozas et al., 1999). During postnatal development, Fra-1 is expressed in all of the main fiber tracts in the cerebrum, whereas in adult rats, Fra-1 activity is mainly confined to the hippocampus. Following brain injuries in adult rats, Fra-1 is found to be highly expressed in the nuclei of reactive astrocytes, suggesting that the transcription factor participates in the activation of target genes (i.e. CD44) that function in repairing brain lesions (Pozas et al., 1999).

Fra-1 is necessary for formation of the placenta (Eferl et al., 2004; Schreiber et al., 2000). In order to determine the function of Fra-1 in development, Schreiber et al. developed Fra-1 knockout mice via gene targeting. Fra-1 knockout mice die *in utero* due to placental defects during embryogenesis. The expression of Fra-1 has been shown to be required for placental vascularization (Schreiber et al., 2000). If the fetus is not receiving adequate blood flow, it is not getting enough nutrients or oxygen to remain alive. After learning that conventional Fra-1 knockout embryos have extraembryonic defects leading to embryonic lethality, Eferl et al. developed conditional Fra-1 mice in order to define the functions of Fra-1 postnatally. The conditional Fra-1 knockout mice were viable and exhibited no major defects as adults, indicating that the transcription factor is nonessential for most organs. The mice did however, develop osteopenia, which is a disease characterized by low bone density These results suggest that Fra-1 functions in bone matrix production by osteoblasts and chondrocytes (Eferl et al., 2004).

2.8 The Oncogenic Function of Fra-1

Increased levels of AP-1 activity have been detected in multiple human cancers, which suggest its role in tumor progression and maintenance (Debinski & Gibo, 2005, 2011; Young & Colburn, 2006). Several studies have shown that Fra-1 plays an essential role in cellular transformation and carcinogenesis. The overexpression of Fra-1 mRNA and protein has been detected in multiple types of human cancers including lung, colon, breast, prostate, and brain cancer (Young & Colburn, 2006). Fra-1 plays a major role in cancer by regulating the expression of its target genes. The binding of the transcription factor to its target genes is what promotes the progression of cancer (Table 2.1) (Verde et al., 2007).

Table 2.1.

Major Roles of Fra-1 and Implicated Target Genes

Fra-1 Function In Tumorigenesis	Target Genes
Cell Proliferation	ccdn1, ccna2, $p19^{ARF}$
Anti-apoptosis	Unknown
Cell migration and Invasiveness	CD44, c-Met, MMP1,MMP9, uPA
Angiogenesis	VEGF-A, VEGF-D

It has been shown that overexpression of Fra-1 contributes greatly to thyroid cell transformation . By inhibiting production of the Fra-1 protein via transfection with an antisense construct, the malignant phenotype of transformed thyroid cells is significantly reduced (Chiappetta et al., 2000). This indicates that Fra-1 plays an essential role in the transformation of cancer cells. The expression of Fra-1 also stimulates transformation and increases the invasiveness and motility of epitheloid adenocarcinoma cells. The cellular transformation of epitheloid cells was also accompanied by the transcriptional activation of genes associated with tumor progression including targets of Fra-1, matrix metallopeptidase 9 (*mmp9)* and urokinase-type plasminogen activator (*uPA)* (Kustikova et al., 1998). The expression of Fra-1 has also been shown to cause a more malignant phenotype in breast tumor cells. Increased levels of Fra-1 expression correlate with an increased expression of genes that have been implicated in cancer progression. Similar to what is seen in epithelial cancers, these genes include Fra-1 target genes, *mmp9* and *VEGF* (Belguise et al., 2005). Increased levels of Fra-1 expression and activity promote cell proliferation and invasiveness of breast cancer. Ramos-Nino et al. showed that the

transformation of mesothelial cells also requires increased Fra-1 expression (Ramos-Nino et al., 2003).

Debinski and Gibo have shown that Fra-1 modulates the malignant features of glioma cells as well (Debinski & Gibo, 2005). GBM cells have a large, flat, and veil-like appearance, compared to normal epithelial cells, which are small and plump. Glioma cells are also resistant to cytotoxic drugs and undergo apoptosis a lot less often than their normal counterparts (Charalambous et al., 2006). Exposing glioma cells to ectopic Fra-1 caused phenotypic and malignant behavior changes in H4 glioma cells, a less aggressive glioma cell line. The H4 glioma cells do not form tumors in nude mice; however, when the H4 cells were introduced to ectopic Fra-1, the cells became tumorigenic (Debinski & Gibo, 2005). Along with the phenotype of H4 glioma cells, the genotype also changes after exposure to ectopic Fra-1. Following exposure, several genes were found to be overexpressed including insulin-like growth factor 3 (*IGF-3*), *IGF-6*, *JunB*, *JunD*, *VEGF*, and early growth response 1 (*EGR1*). Exposure of glioma cells to Fra-1 also resulted in decreased expression of a few genes such as N-cadherin and granulocyte macrophage colony stimulating factor (*GM-CSF*) (Debinski & Gibo, 2005). The overexpression of Fra-1 in glioma cells and in rat fibroblasts stimulates anchorage-independent growth, which is a main trait distinguishing tumor cells from their normal counterparts (Chiappetta et al., 2007; Debinski & Gibo, 2005). Transfection of U-87 GBM cell lines with sense (+) and antisense (-) Fra-1 alters the morphology and tumor formation in glioblastoma multiforme. Compared to U-87 parentals, cells transfected with the antisense Fra-1 transgene were larger, more rounded, and had fewer and/or shorter processes. On the other hand, U-87(Fra-1(+)) cells were more elongated with larger processes. Debinski and Gibo also showed that Fra-1 knockdown via Fra-1 siRNA results in a decrease in U-87 MG tumor size. Compared

to U-87(Fra-1(-)) clones, the tumor size of normal and Fra-1 (+) transfected cells were more than six times larger (Debinski & Gibo, 2005). It has also been shown that a decrease in Fra-1 expression results in a decreased migration rate via wound healing assays of fra-1 siRNA transfected glioma cells (Debinski & Gibo, 2011). These results suggest that the transcription factor plays a major role in glioma cell transformation, tumor growth, and migration.

The AP-1 transcription factor, Fra-1, has been shown to regulate the expression of CD44 in glioma cells. Fra-1 expression and activity has also been shown to promote CD44-mediated GBM cell adhesion (Raines, 2010). Adhesion is necessary for the migration and invasion of tumor cells into healthy surrounding tissue; therefore, we must investigate the role of Fra-1 regulated CD44 expression in the invasive migratory behavior of glioblastoma multiforme in order to improve the treatment and prognosis of the disease.

2.9 Research Objectives

The overall objective of the study is to determine whether Fra-1 mediated regulation of CD44 expression affect cellular migration in Glioblastoma multiforme. The hypothesis of this thesis was that overexpression of Fra-1 leads to increased CD44 expression, promoting the migratory capacity of GBM cells. The hypothesis of this thesis was based off conclusions from previous studies, which focused on the role of Fra-1 and CD44 in cancer. Increased levels of Fra-1 and CD44 have been detected in multiple human cancers, which suggest their roles in tumor progression and maintenance (Yoshida et al., 2012; Young & Colburn, 2006). Debinski and Gibo have shown that increased Fra-1 expression contributes to the malignant phenotype of glioma cells (Debinski & Gibo, 2005). In mesothelioma, Fra-1 and CD44 expression were shown to be associated. Furthermore, knockdown of Fra-1 decreased the migration rate of mesothelioma cells (Ramos-Nino et al., 2007; Ramos-Nino et al., 2003). CD44 expression has

also been linked to Fra-1 expression and activity in malignant brain tumor cells. In addition, CD44-mediated cell adhesion of glioma cells has been shown to be promoted by Fra-1 activity (Raines, 2010). In order for tumor cells to migrate, they must adhere to the extracellular matrix. Upon adherence, the tumor cell is stimulated to secrete proteolytic enzymes, which degrade components of the ECM, allowing the cancer cell to migrate and invade surrounding healthy tissue. Based on these conclusions, this thesis has focused on how the modulation of Fra-1 and CD44 affects CD44-associated migration in GBM.

To achieve the goals of this study, the three specific aims were: (1) to determine whether the modulation of Fra-1 expression affects CD44-mediated GBM cell migration; (2) to investigate whether Fra-1 regulates CD44 expression through a MAPK and/or AKT-mediated mechanism; (3) and to examine whether decreased expression of Fra-1 via pathway inhibition prevents glioma cell migration. The findings of the study could lead to a better understanding of how Fra-1 regulated expression of CD44 affects GBM cell migration, and to the identification of pathways that could be targeted to combat the aggressiveness and improve the prognosis of GBM.

CHAPTER 3

Materials and Methods

3.1 Culture and Passage of Glioma Cells

The A172 GBM parentals and clones were a kind gift from Dr. Waldemar Debinski of Wake Forest University, Winston-Salem, NC. The cells were grown in Dulbecco's Modified Eagle High Glucose 1X Medium (DMEM) (Gibco®), 10% fetal bovine serum (FBS) (Hyclone®), and 100 µg/mL Penicillin-Streptomycin 1X Solution (Cellgro®). Each cell line was rinsed with Dulbecco's Phosphate Buffered Saline (DBPS) (Gibco®). The A172-mw12 and A172-mw6 cell lines were treated with 50 µg/mL G418 Sulfate Solution (Cellgro®) in order to ensure that all cells contained the plasmid. Table 3.1 highlights differences between the A172 clones.

Table 3.1.

A172 GBM Clones

Cell Line	Description
A172-wt	Contains no vector; Normal Fra-1 expression levels
A172-v43	Contains empty vector; Normal Fra-1 expression levels
A172-mw6	Contains dominant negative vector that results in decreased Fra-1 expression levels
A172-mw12	Contains vector that results in increased Fra-1 expression levels

3.2 Western Blot Analysis

Cell lysates were extracted with 1% Trition-X100 (Sigma) and 0.2% Nonidet P-40 (NP-40) (Sigma) in the presence of a protease inhibitor (Sigma). Lysates containing proteins were separated by SDS-PAGE using 8% polyacrylamide gels. The proteins were then transferred to

Polyvinylidene Fluoride (PVDF) (Fisher) and blocked for at least one hour with 5% milk in PBS containing 0.05% Tween 20. Membranes were incubated with primary antibodies overnight at 4^{o}C while shaking. The primary antibodies were specific for c-Met, Fra-1 and phosphorylated Fra-1 (Santa Cruz Biotechnology), and CD44 (R&D Systems). B-tubulin (Sigma) or actin (WFU) was used as loading controls. Primary antibodies were diluted in 5% milk PBS containing 0.05% Tween 20 as follows: Fra-1 at a 1:1,000 dilution; CD44 at a 1:5,000 dilution; and β-tubulin or actin at a 1:5,000 dilution. Following primary antibody incubation, the membrane was washed three times at 10 minutes each with PBS containing 0.05% Tween 20. The membrane was then incubated with a secondary antibody conjugated with horseradish peroxidase, goat anti-mouse IgG (Sigma) or goat anti-rabbit IgG (Sigma), at a dilution of 1:2,000 and 1:2,000, respectively, in 5% milk in PBS containing 0.05% Tween 20 for one hour. Following secondary antibody incubation, the membrane was washed three times for 10 minutes each in PBS containing 0.05% Tween 20. Detection of protein bands was carried out using x-ray film radiography.

3.3 A172 Cell Migration Assay

The appropriate wells of a 24-well plate were coated with 300 µL of hyaluronic acid (1mg/mL). For two hours, the plate was left to air dry under the tissue culture hood. The 24-well plate was then placed into the 4^{o}C refrigerator and left overnight. Following incubation, each well was blocked with 300 µL of heat inactivated bovine serum albumin (500 µg/mL) for 30 minutes in a 37^{o}C incubator. The heat-inactivated BSA was then removed, and the plates were washed twice with 300 µL of BSA (100 µg/mL). The A172 MG clones (A172-wt, A172-v43, A172-mw6, and A172-mw12) expressing various levels of Fra-1 were then seeded into each well and allowed to reach confluence. Using a 200 µL pipette tip, a wound was scratched down

the middle of each well. After wounding, the cells were cultured for 24 hours. Pictures were taken at 0hr, 18hr, and 24hr time frames. The percent closure of the wound between 0hr and 24hr was calculated for each A172 clone using the following equation:

Percent Closure = 100 - [Average wound width at 24hr / Average wound width at 0hr x 100]

3.4 A172 Pathway Inhibition Migration Assay

The appropriate wells of two 6-well plates were filled with 300 mL of DMEM containing serum and plated with cells that they would be approximately 80% confluent after 24 hours. After 24 hours, two wounds were made in each well with a 200 μL pipette tip. After wounding, the media was aspirated off, and the cells were washed twice with PBS. The appropriate treatment was then applied to each well. Pathway inhibitors were diluted with plus serum DMEM at a 1:1,000 dilution. U0126 and LY294002 were used at a concentration of 10 μM and 20 mM, respectively. After treatment, the cells were then cultured for another 24 hours. Pictures of each wound were taken at 0hr and 24hr time frames after treatment with U0126 or LY294002. The percent closure of the wound between 0hr and 24hr was calculated for each A172 clone using the equation described above.

3.5 Statistical Analysis

All data was processed using Prism® software. Duplicate determinations were used per group, and results were evaluated by one-way analysis of variance using the Newman-Keuls procedure for comparisons between treatment groups. Differences with p values < 0.05 were considered statistically significant.

3.6 Quantification of Western Blots

Densitometry was used to measure the relative absorbance values for each band from western blot analysis. Adobe® Photoshop (PS) software. The western blot data was loaded onto

PS. The image was inverted and a box was drawn around one band at a time with the Marquee Tool. A histogram was then used to obtain the mean and pixel values for each band. To calculate the absolute intensity, the value of the mean and pixels were multiplied together. The relative intensity value was calculated by dividing the absolute intensity value of each cell line or treatment by the corresponding absolute intensity value of the loading control (tubulin or actin).

CHAPTER 4

Results

4.1 Examine Fra-1 and CD44 Protein Expression Levels in A172 Clones

Western blot analysis was performed in order to ensure that the A172 clones expressed various levels of Fra-1 expression. A172-mw6 cells, which contain a dominant negative Fra-1 knockdown vector, exhibited decreased Fra-1 expression levels compared to the A172-wt and A172- v43 cells that contain an empty vector (Figure 4.1). The A172-mw12 cell line contains a plasmid with a mutation causing the GBM cells to overexpress Fra-1. Western blot analysis shows that these cells overexpress Fra-1 when compared to the A172 and A172-v43 cell lines (Figure 4.1) (Debinski & Gibo, 2005).

In order to determine whether a decrease in Fra-1 expression subsequently lead to a decrease in the expression of CD44, the A172-wt and A172-mw6 cells were probed for CD44 and analyzed. Compared to the A172-wt cell line, the A172-mw6 cells exhibited a lower expression of Fra-1 as well as CD44 (Figure 4.1 and Figure 4.2).

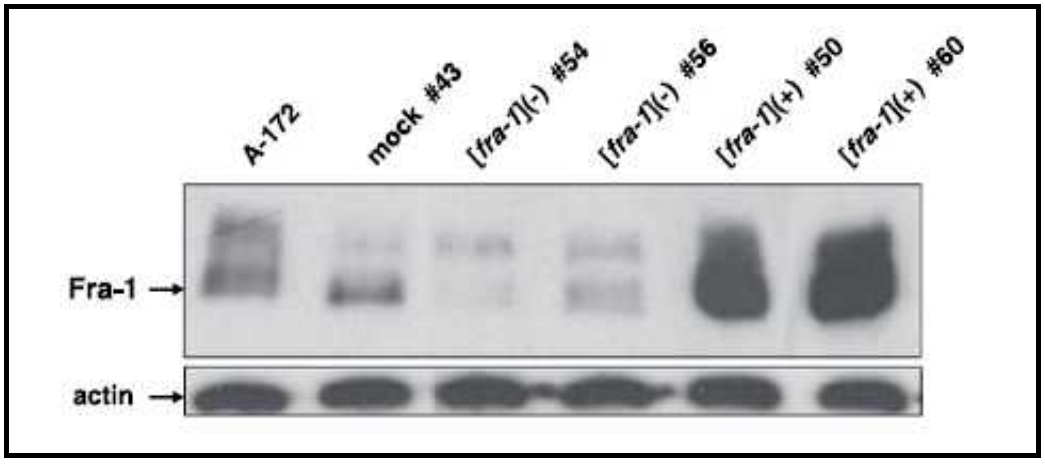

Figure 4.1. Fra-1 expression in A172-wt and clones.

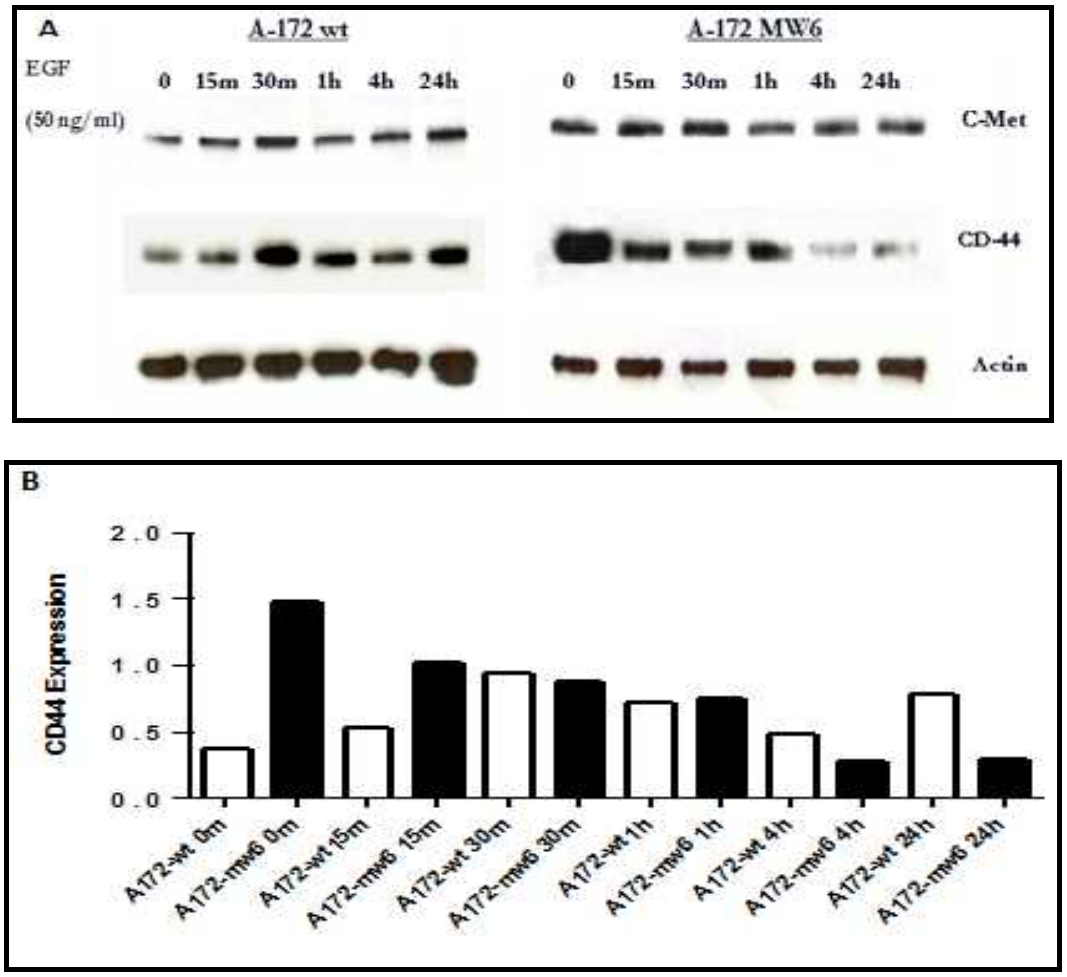

Figure 4.2. CD44 expression in A172-wt and A172-mw6 cell lines.

4.2 Examine the Effect of Fra-1 Modulation on GBM Cell Migration

Many studies have shown that Fra-1 promotes tumor cell migration in a number of different cancer types (Belguise et al., 2005; Kustikova et al., 1998; Luo et al., 2009; Sayan et al., 2011; Young & Colburn, 2006). In the following experiment, the A172 cell line and its vector-containing clones (A172-v43, A172-mw6, and A172-mw12) were utilized to examine how Fra-1 expression levels affect the migration of GBM cells (Figure 4.3). The A172-mw6 (Fra-1 dominant negative) cells exhibit a lower percent closure after 24 hours, and therefore a slower migration rate compared to the A172-v43 controls and the parental A172 cell line. The A172-mw12 cell line (Fra-1 overexpressors) exhibits a higher percent closure compared to

controls. The percent closure of the A172-mw12 cell line is more than twice that of the A172-mw6 cells.

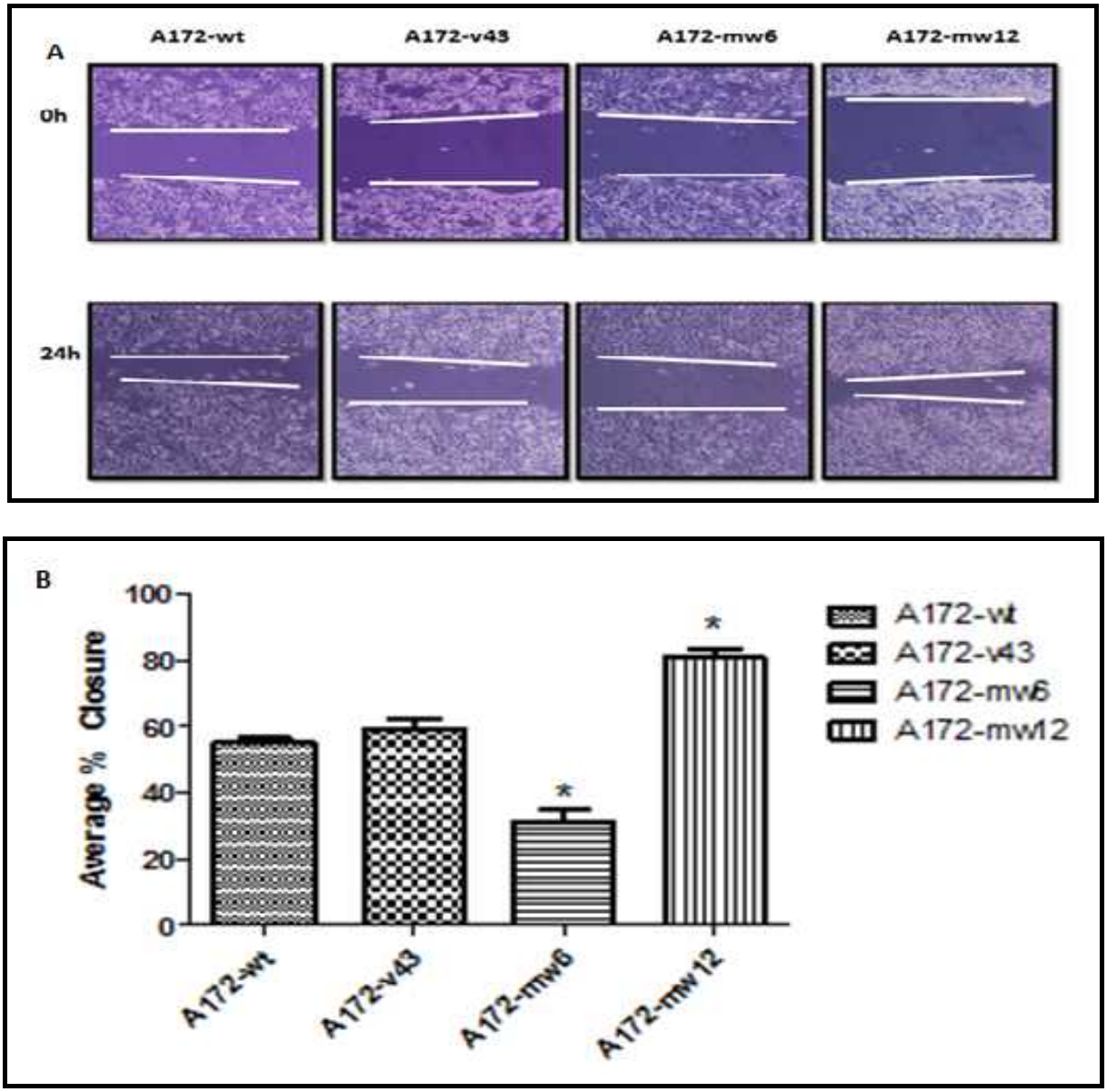

Figure 4.3. Modulation of Fra-1 affects A172 GBM cell migration on HA.

4.3 Identification of Pathways Responsible for Fra-1 Regulated CD44 Expression

In order to determine which signal transduction pathways were responsible for Fra-1 mediated expression of CD44 in GBM, A172 parental cells were treated with inhibitors of the AKT/mTOR and the MAPK pathway. Studies have shown that Fra-1 is an AKT-inducible gene

in both mesothelioma and prostate cancer (Ramos-Nino et al., 2007). The PI3K/AKT/mTOR pathway is capable of regulating a number of cellular functions that promote tumorigenicity including cell survival, growth, proliferation, as well as inhibiting pro-apoptotic activity (Tiwari et al., 2003). Fra-1 expression and its activation by the MAPK pathway have been well documented in several cancers including colon, lung, and mesothel9ioma (Adiseshaiah et al., 2005; Jones et al., 2000; Ramos-Nino et al., 2007). The MAPK signal transduction pathway also plays a role in cellular survival, growth, proliferation, migration, cell cycle regulation, and angiogenesis. All of these cellular functions, when gone awry, play a major role in the development of gliomas.

The stimulation of A172 parental cells with EGF (20 ng/mL) results in an increase of total Fra-1, phosphorylated Fra-1, and CD44 expression at 4 and 24 hours (Figure 4.4). A172-wt cells treated with EGF in the presence of LY294002 (20 μM), a phosphatidylinositol 3-kinase inhibitor (PI3K) exhibited a decrease in total Fra-1, phosphorylataed Fra-1, and CD44 expression levels compared to the EGF-treated controls (Figure 4.4A and Figure 4.4C). Cells treated with EGF in the presence of rapamycin (25nM), an mTOR pathway inhibitor, also exhibited a decrease in total and phosphorylated Fra-1 and CD44 expression (Figure 4.4A and Figure 4.4C). A172 GBM cells were also treated with a MEK1/2 inhibitor and MEK1 inhibitor, U0126 (25 μM) and PD 98059 (10 μM), respectively (Figure 4.4B and Figure 4.4D). Cells treated with the MAPK pathway inhibitors exhibited a decrease in phosphorylated Fra-1 expression; however, MAPK inhibition had little to no effect on decreasing total Fra-1 and CD44 expression levels (Figure 4.4B and Figure 4.4D).

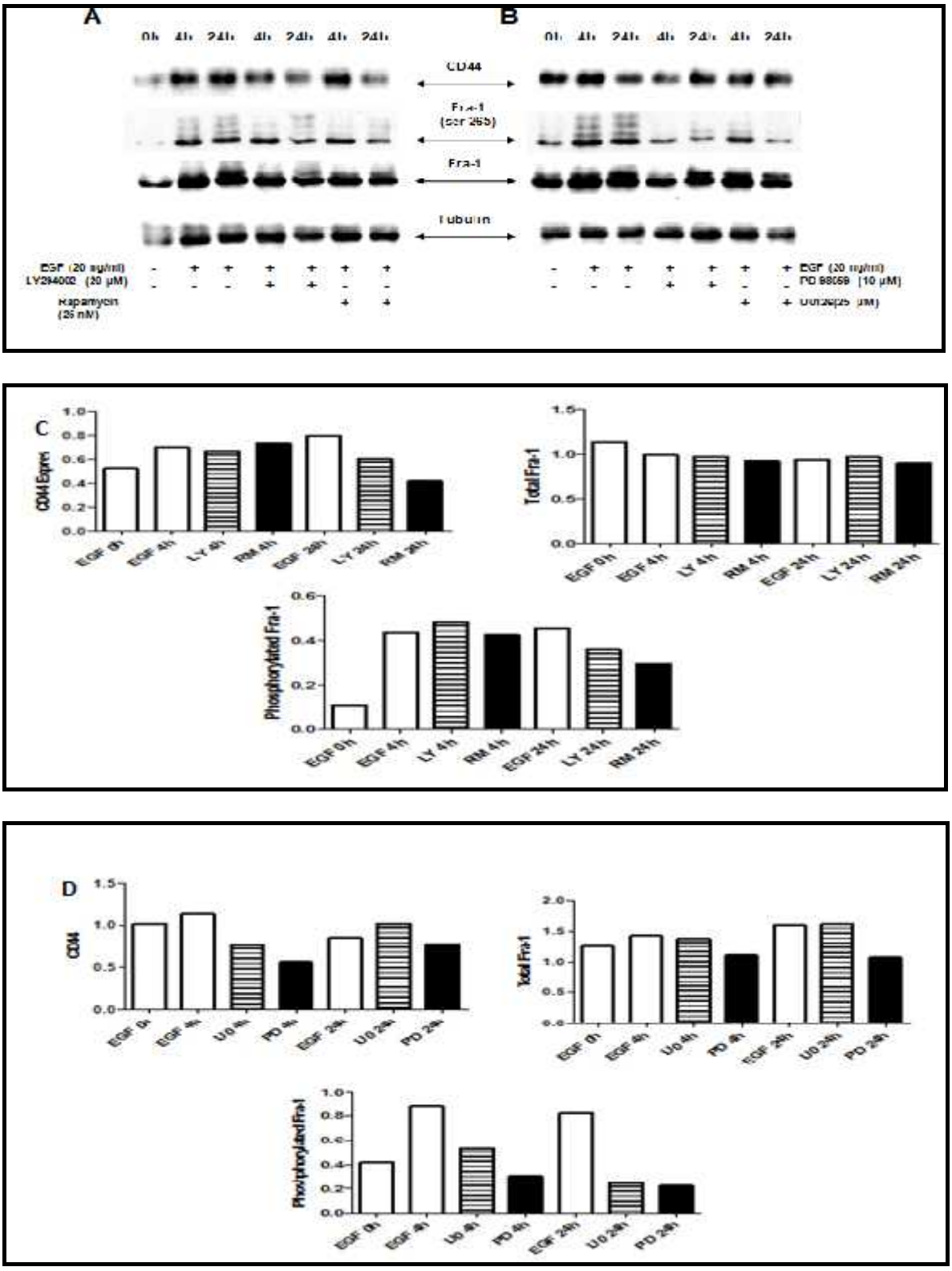

Figure 4.4. Fra-1 and CD44 expression in A172-wt cells following exposure to MAPK and AKT/mTOR inhibitors.

4.4 Pathway Inhibition Affects GBM Cell Migration

Western blot analysis revealed that AKT/mTOR pathway inhibition decreases total and phosphorylated Fra-1expression as well as CD44 expression in glioma cells. It also revealed that MAPK inhibition decreases phosphorylated or active Fra-1, although it has little to no effect on total Fra-1 or CD44 expression levels (Figure 4.4). Therefore, the next step was to determine whether Fra-1 knockdown via pathway inhibition affected GBM cell migration. A172 parental cells treated with plus serum media in the presence of U0126 (MEK1/2 inhibitor) exhibited a slight decrease in cellular migration compared to the control cells, treated with plus serum media. A172 parental cells showed a decreased rate of migration in the presence of the AKT inhibitor, LY294002 (Figure 4.5).

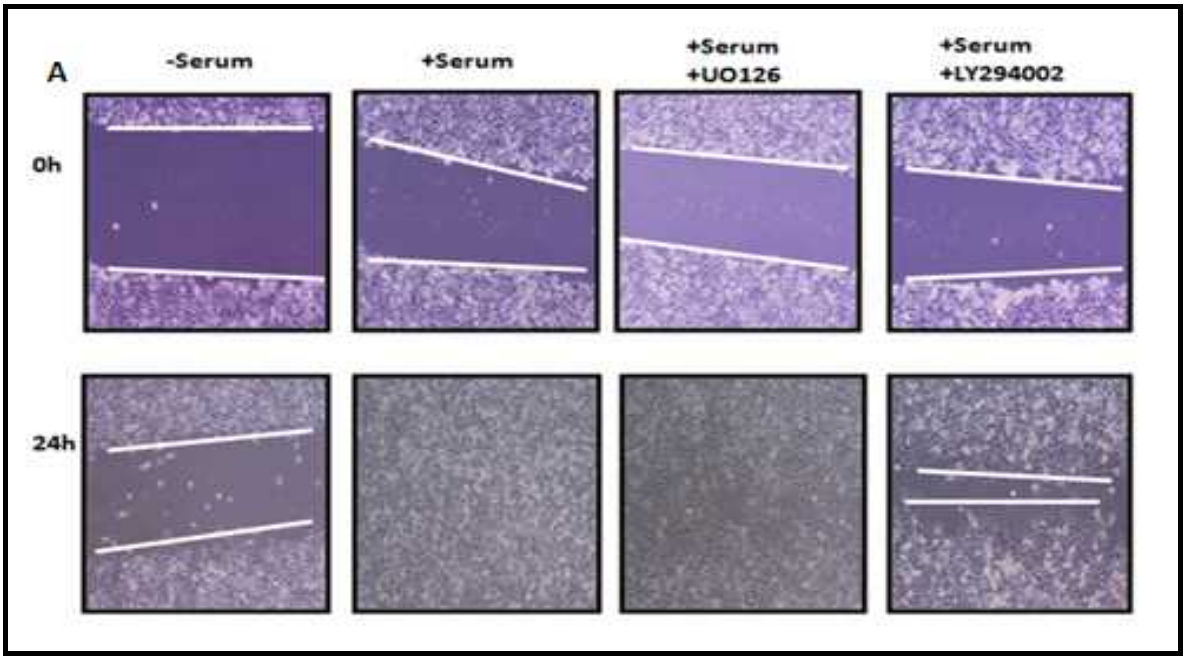

Figure 4.5. Fra-1 knockdown via MAPK and AKT/mTOR inhibition affects GBM migration.

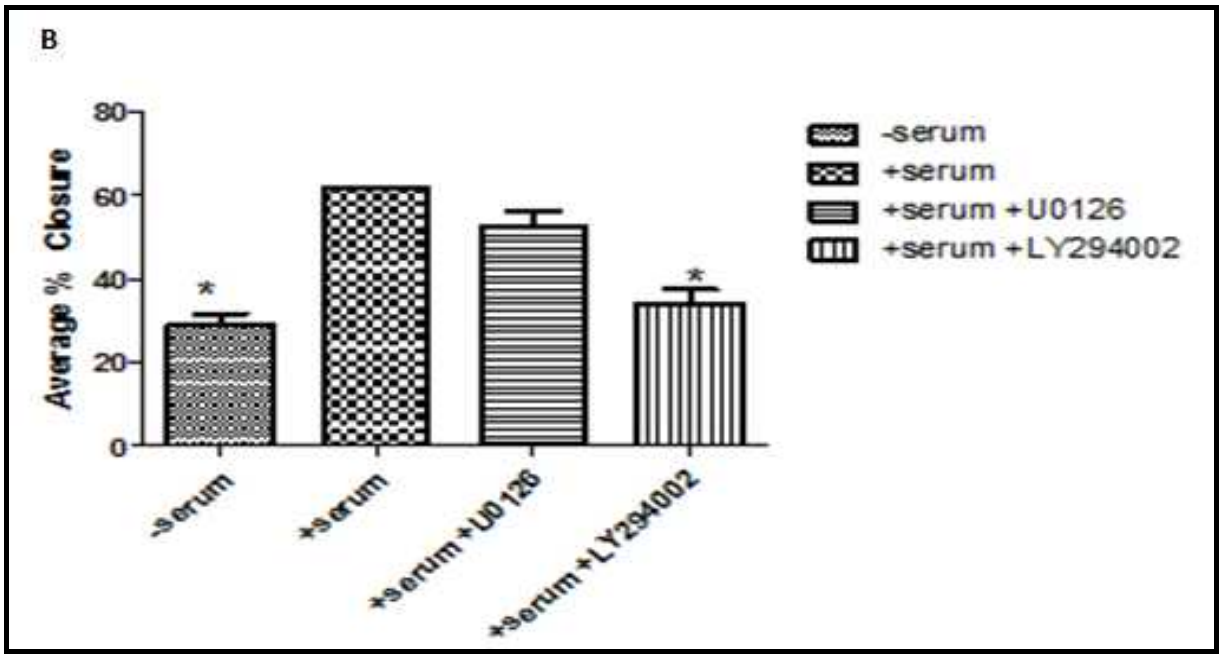

Figure 4.5. cont.

CHAPTER 5

Discussion

Glioblastoma multiforme (GBM) is a highly aggressive, grade IV, primary brain tumor characterized by the highest proliferation rate and the highest capacity to invade surrounding health tissue (Helseth et al., 2010; Wei et al., 2010). Unfortunately, GBM is often resistant to or develops resistance against current treatment, which is surgical resection followed by chemotherapy and/or radiation treatment. The invasive migratory nature of glioma cells is the major driving force behind the aggressiveness of GBM, resulting in a very poor prognosis. There are many underlying factors that regulate glioma cell migration, allowing them to metastasize. The interaction between adhesion molecules present on tumor cells with ECM components results in the degradation of the ECM. This allows the glioma cell to migrate and spread to healthy tissue (Claes et al., 2007). The adhesion molecule CD44 along with the transcription factor, Fra-1, were the primary focus of this study. Specifically, the overall objective of this thesis was to investigate how Fra-1 mediated regulation of CD44 affects the migratory capacity of GBM cells. CD44 was first shown to be linked to Fra-1 in an equally aggressive cancer, mesothelioma (Ramos-Nino et al., 2007). A former member of the Martin laboratory later showed that CD44 expression correlates with Fra-1 expression in GBM as well (Raines, 2010).

In this study, the regulation of CD44 expression by Fra-1 was reaffirmed via western blot analysis using A172 MG clones that expressed various levels of Fra-1. Western blot analysis was also performed on the A172 parental cell line in order to identify cell signaling pathways responsible for Fra-1 mediated regulation of CD44. Wound healing assays were conducted on A172 clones to demonstrate that CD44-mediated cell migration was regulated by Fra-1, and on

A172 parentals to demonstrate the effect of MAPK and AKT/mTOR pathway inhibition on the migration of glioma cells.

5.1 Expression Levels of Fra-1 and CD44 in GBM cells

Debinski and Gibo used western blot analysis to demonstrate that the A172 GBM clones expressed various levels of Fra-1 (Figure 4.1). In Figure 4.2, CD44 expression is examined in the A172-wt and A172-mw6 cell line. Compared to the A172-wt cells that express normal Fra-1 levels, the A172-mw6 (Fra-1 dominant negative) cell line exhibits a lower level of CD44 expression. The data from the study shows that decreased expression of Fra-1 correlates with decreased expression of CD44. These results were anticipated since it has previously been demonstrated that Fra-1 knockdown via siRNA in additional glioma cell lines (U-251 MG and U-1242 MG) results in decreased expression of CD44 (Raines, 2010).

5.2 CD44-Mediated GBM Cell Migration is Regulated by Fra-1 Expression

After determining that Fra-1 expression correlates with CD44 expression in the A172 clones, this study examined whether CD44-mediated migration of GBM cells is regulated by the AP-1 transcription factor. In mesothelioma, both Fra-1 and CD44 are linked to cell migration. Ramos-Nino et al. showed that a decrease in Fra-1 and CD44 expression, via shRNA constructs, lead to a decrease in the migratory capacity of mesothelioma cells (2007). These results suggest that Fra-1 regulates the migration of mesothelioma cells through modulation of CD44 expression (Ramos-Nino et al., 2007). Through the use of Fra-1 siRNA, knockdown of Fra-1 has also been shown to lead to a decrease in the cellular migration of A172 parental cells (Debinski & Gibo, 2011). It has also been shown that Fra-1 knockdown reduces tumor cell migration in breast and bladder cancer (Luo et al., 2009; Sayan et al., 2011).

This was the first study to examine whether an increase in Fra-1 expression increases the migration rate of GBM cells. When Fra-1 is overexpressed, as in the A172-mw12 cell line, the migration rate of glioma cells increases (Figure 4.3). Similar to a previous study, a decrease in Fra-1 results in a decrease of the migratory capacity of glioma cells (Debinski & Gibo, 2011). The results of this study have shown that a decrease in Fra-1 expression correlates with decreased CD44 expression, leading to a decrease in the migratory capacity of brain tumor cells. Collectively, these results suggest that the modulation of Fra-1 affects CD44-mediated migration of glioma cells.

5.3 MAPK and AKT/mTOR Inhibition Decreases Fra-1 and CD44 Expression

In this thesis, we focused on whether Fra-1 expression in human GBM cells is regulated by the AKT/mTOR and MAPK cell signaling pathways. In Figure 4.4A, inhibition of the AKT/mTOR pathway using the PI3K inhibitor, LY294002 (20 μM) and the mTOR inhibitor, rapamycin (25 nM), caused a reduction of overall Fra-1, phosphorylated Fra-1, and CD44 expression in A172 GBM cells. Glioma cells treated with U0126 (25 μM), a MEK 1/2 inhibitor, and PD98059 (10 μM), a MEK1 inhibitor, also exhibited decreased expression of phosphorylated Fra-1 (Figure 4.4B); however, there was minimal change in the expression levels of total Fra-1 and CD44. The results gathered in the study agree with findings in mesothelioma. Inhibition of PI3K and MEK1 with LY294002 (20 μM) and PD98059 (30 μM), respectively, caused significant reduction of Fra-1 mRNA levels in numerous mesothelioma cell lines (Ramos-Nino et al., 2007). The results of this research suggest that CD44 expression is regulated by Fra-1 in an AKT/mTOR dependent manner, and that MAPK regulated Fra-1 has virtually no effect on CD44 expression in GBM cells. In GBM, AKT/mTOR regulated Fra-1 appears to stimulate the transcription of CD44, which in turn greatly affects the migratory

capacity of glioma cells. It is also possible that AKT-regulated Fra-1 activates transcription of other genes involved in cell migration and invasion such as *uPA/uPAR*, *MMP3*, and *MMP9* (Kustikova et al., 1998). Studies have shown that MAPK inhibition decreases cellular proliferation in glioma cells, and it is believed that inhibition of MAPK affects the proliferative capacity of glioma cells more than other cellular functions (Huang et al., 2009; Zohrabian et al., 2009). Therefore, MAPK regulated Fra-1 may activate the transcription of genes that promote tumor cell proliferation (Kustikova et al., 1998).

5.4 Fra-1 Knockdown Via Pathway Inhibition Decreases GBM Migration

After observing that MAPK and AKT/mTOR pathway inhibition decreases Fra-1 phosphorylation (Figure 4.4), the next step was to identify whether this knockdown affected the migratory capacity of human GBM cells since both pathways have been implicated in cellular migration (Hennessy et al., 2005). In order to determine if pathway inhibition affects glioma cell migration, wound healing assays were performed on the A172 parental cell line. The results indicate that MAPK and AKT pathway inhibition decreases the migratory capacity of glioma cells (Figure 4.5). However, AKT inhibition had a more pronounced effect on decreasing cellular migration, whereas MAPK inhibition resulted in only a slight decrease when compared to controls. The average percent closure after 24 hours of the control cells treated with plus serum media was similar to that of cells treated with the MAPK inhibitor, U0126. However, inhibition of the AKT/mTOR pathway via LY294002 resulted in a percent closure of that was significantly lower compared to the controls (Figure 4.5B). Generally, MAPK is the dominant signaling pathway associated with proliferation, whereas the AKT pathway is usually involved in tumor cell invasion (Nakada et al., 2011). These results suggest that AKT-regulated Fra-1 may play a bigger role in migration, and that MAPK-regulated Fra-1 plays more of a prominent role

in the proliferation of glioma cells. This thesis proposes that MAPK and AKT/mTOR-regulated Fra-1 may activate transcription of distinct gene subsets involved in either proliferation or migration, respectively.

CHAPTER 6

Conclusion

The research of this thesis confirms that the AP-1 transcription factor, Fra-1, and the cell surface protein, CD44, play an important role in glioma cell migration. Overexpression of Fra-1 and CD44 is characteristic of many tumor cell types. A wound healing assay confirmed that increased Fra-1 expression promotes CD44-mediated migration of human GBM cells. This study has also demonstrated that both MAPK and AKT/mTOR pathway inhibition decreases Fra-1 phosphorylation. However, only AKT inhibition results in noticeable decreases of total Fra-1 and CD44 expression levels. It has also been shown that AKT inhibition significantly decreases glioma cell migration, whereas MAPK inhibition only slightly decreases the migratory capacity of GBM cells.

It has now been shown that Fra-1 regulates not only CD44-mediated adhesion of glioma cells, but migration as well. However, more research must be done in order to determine whether Fra-1 is directly linked CD44 expression in Glioblastoma multiforme. This could be demonstrated via a chromatin immunoprecipitation (ChIP) assay, which would be used to determine whether CD44 expression is directly linked to Fra-1 by examining whether the transcription factor actually binds to the promoter region of CD44, leading to its activation and regulation. Future studies should also focus on determining the role of MAPK-regulated Fra-1 in GBM. An MTT assay could be conducted in order to examine whether MAPK regulated Fra-1 plays a major role in glioma cell proliferation. Real-time PCR studies may also be conducted in order to determine whether mRNA expression of CD44 is regulated by Fra-1 as well.

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Appendix

World Health Organization Grading Scheme

Grade	Characteristics	Tumor Types
I	▪ Least malignant ▪ Grow slowly ▪ Cell appear almost normal under microscope ▪ Often associated with long-term survival	▪ Gangliocytoma ▪ Pilocytic astrocytoma
II	▪ Relatively slow growing ▪ Cells appear slightly abnormal under microscope ▪ May spread into healthy surrounding tissue ▪ May recur as a higher grade tumor	▪ Pineocytoma ▪ Pure oligodendroglioma
III	▪ Malignant ▪ Actively reproduces abnormal cells ▪ Cells appear normal under microscope ▪ Spreads to surrounding healthy tissue ▪ Tends to recur, often as a higher grade tumor	▪ Anaplastic astrocytoma ▪ Anaplastic oligodendroglioma
IV	▪ Most malignant ▪ Actively reproduces abnormal cells ▪ Cells appear very abnormal under microscope ▪ Easily spreads into healthy surrounding tissue ▪ Angiogenesis occurs rapidly ▪ Necrosis	▪ Glioblastoma multiforme (GBM) ▪ Medulloblastoma