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Developing Epithelial Membrane Protein 2 as a Prognostic Marker for Glioblastoma Treatment Response and Outcomes: Study Proposal and Design

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Publication Date

2017

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**University of California,
Irvine**

**Developing Epithelial Membrane Protein 2 as a Prognostic Marker for
Glioblastoma Treatment Response and Outcomes: Study Proposal and Design**

THESIS

submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in Biomedical and Translational Science

by

Panayiotis E. Pelargos

**Thesis Committee:
Professor Sheldon Greenfield, MD, Chair
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2017

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List of Abbreviations

BBB = Blood-brain barrier

CBC = Complete blood count

CNS = Central nervous system

DAB = 3,3'-Diaminobenzidine

EGFR = Epidermal growth factor receptor

EMP2 = Epithelial membrane protein 2

FAK = Focal adhesion kinase

GAS3/PMP22 = Growth arrest-specific gene 3/peripheral myelin protein-22

GBM = Glioblastoma

GLUT-1 = Glucose transporter 1

HIF-1 = Hypoxia-inducible factor 1

HUVEC = Human umbilical vein endothelial cells

IDH1 = Isocitrate dehydrogenase 1

Ig = Immunoglobulin

MGMT = O-6-methylguanine-DNA methyltransferase

MRI = Magnetic resonance imaging

NCI-EDRN = National Cancer Institute – Early Detection Research Institute

Ribo = Ribozyme

shRNA = Short hairpin ribonucleic acid

siRNA = Small interfering ribonucleic acid

TMA = Tissue microarray analysis

TP53 = Tumor protein p53

U-87 MG = Uppsala 87 malignant glioma

VEGF = Vascular endothelial growth factor

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ABSTRACT OF THE THESIS

Developing Epithelial Membrane Protein 2 as a Prognostic Marker for
Glioblastoma Treatment Response and Outcomes: Study Proposal and Design

By

Panayiotis E. Pelargos

Master of Science in Biomedical and Translational Science

University of California, Irvine 2017

Professor Sheldon Greenfield, MD, Co-Chair

Professor Sherrie H. Kaplan, PhD, Co-Chair

Background: Glioblastoma is a highly aggressive brain cancer that leads to death within 15 months of diagnosis. Despite active research in the field, effective treatment options are currently limited to surgery, radiotherapy, and chemotherapy. Recently, there has been a push towards characterizing glioblastoma tumors genetically, epigenetically, transcriptionally, and proteomically in order to develop new, more targeted and individualized therapeutic approaches to glioblastoma.

Aims: The aim of this thesis is to develop a study proposal for the characterization of one of the genes believed to be over-expressed in glioblastoma, EMP2. The study proposed will evaluate EMP2 role's in glioblastoma pathogenesis and

evaluate the therapeutic potential of antibodies developed against EMP2 expressed on the glioblastoma surface membrane.

Methods: *In-vitro* and *in-vivo* background studies performed in our labs as well as those found in the literature are presented and discussed as the foundation and groundwork for the proposed study. The study proposal is then outlined in detail.

Results: The purpose of the proposed study is to demonstrate the efficacy of targeting EMP2 on glioblastoma tumor cells using an antibody against EMP2. A detailed description of the proposed study design is given followed by the dissemination and implementation potential of the results of this study. We hypothesize that the functional consequence of EMP2 up-regulation in glioblastoma cells is to orchestrate the membrane expression of proteins required for cell migration, invasion, and neoangiogenesis. Additionally, we predict that the expression of EMP2 can be therapeutically exploited as a novel treatment target using antibodies against EMP2.

Discussion: Our results from the background studies and the expected results from the study proposal suggest that EMP2 may be a promising molecular therapeutic target in glioblastoma. Additional work will be needed to determine whether anti-EMP2 antibodies can be combined with standard chemotherapy or molecularly-targeted treatments to treat glioblastoma in human patients.

Introduction

Glioblastoma (GBM) is a high-grade (stage IV) glioma cancer. GBM is the most common and aggressive form of primary brain tumor, with a median survival of only 15 months with current treatment strategies.[1] GBM cells are highly invasive which lends to their rapid and deadly disease course.[2] In contrast, low-grade (stage I-III) gliomas, while having the potential to progress to GBM, have a milder disease course.

Epithelial membrane protein-2 (EMP2) is a member of the growth arrest-specific gene 3/peripheral myelin protein-22 (GAS3/PMP22) subfamily, which together with tetraspanins and connexins, comprise the three subfamilies of the large 4-transmembrane family.[3] Its expression is increased in breast, ovarian, and endometrial cancers where it has been shown to correlate with poor survival and/or advanced disease.[4-7] EMP2 is expressed at low or undetectable levels in normal brain tissue.[2] In GBM, EMP2 mRNA is up-regulated and EMP2 is highly expressed on the cell surface.[2, 8] In GBM, it enhances tumor growth *in-vivo* partly by up-regulating $\alpha v\beta 3$ integrin surface expression, activating focal adhesion kinase and Src kinase, and promoting cell migration and invasion.[2] EMP2 expression is significantly correlated with activated Src kinase in patient samples and promoted tumor cell invasion using intracranial mouse models.[2] In intracranial mouse models, EMP2 also promoted tumor cell invasion and a more aggressive disease phenotype.[2]

In the following thesis, I will outline a grant proposal for a study aimed at elucidating the role of EMP2 in GBM pathogenesis as well as investigating the potential for EMP2 to be therapeutically exploited as a novel treatment target using antibodies against EMP2 in GBM. I will first outline GBM pathology and where are currently in terms of outcomes and treatment options. I will then present a background of EMP2, its discovery, its cellular function, and how it

is being targeted in other tumors. Finally, I will outline a study proposal for the elucidation of EMP2's role in GBM pathogenesis and to demonstrate the efficacy of targeting EMP2 on GBM tumor cells using an antibody against EMP2.

Chapter 1

Background

GBM Background

Gliomas are tumors made up of neoplastic glial cells that can arise anywhere along the central nervous system (CNS). Glial cells are non-neuronal cells that are present in both the CNS and peripheral nervous system and include oligodendrocytes, astrocytes, ependymal cells, microglia, Schwann cells, and satellite cells. Gliomas account for approximately 30% of CNS tumors and 80% of all malignant brain tumors.[9] They are classified by the type of cell with which they share histological features, e.g. ependymoma, astrocytoma, or oligodendroglioma, and they are further categorized by grade which is determined pathologically and histologically.[9, 10] Traditionally, histology subtype and tumor grade have been used to determine the malignant potential, treatment response, and survival outcomes.[9-11] Recently, there has been a push towards characterizing GBM tumors genetically, epigenetically, transcriptionally, and proteomically in order to develop new, more targeted and individualized therapeutic approaches to GBM.[12]

GBM Epidemiology

GBM is one of the primary neuroepithelial tumors of the central nervous system. It is classified as a high-grade (stage IV) glioma tumor made up of malignant astrocytes. GBM is the second most common brain tumor and the most common and aggressive form of primary malignant brain tumors in adults making up approximately 15% of CNS tumors, 47% of malignant primary CNS tumors, and 55% of primary CNS gliomas (Figure 1).[1, 12-14] The incidence rate of GBM is 3.2 per 100,000 population.[13]

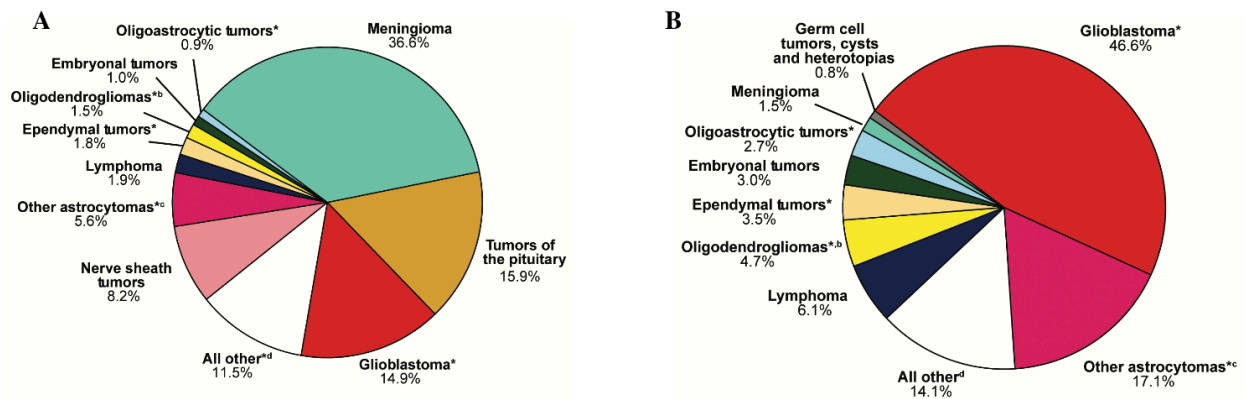


Figure 1. Distribution of CNS tumors. (A) Distribution of all primary CNS tumors by histology. (B) Distribution of all primary malignant CNS tumors by histology. Figure adapted from [13].

GBM can develop either de novo as a primary tumor or progress from a lower grade glioma.[15] Whether it is classified as a primary or secondary GBM typically depends on the clinical presentation of the tumor. Most GBMs (~80%) are primary GBMs.[9, 16] Primary GBMs typically present more quickly with a symptom onset of 3-6 months prior to diagnosis without clinical or histological evidence of a pre-existing lower grade glioma.[9, 16] Secondary GBMs progress more slowly and develop from lower grade gliomas within 4-5 years.[9, 16] Additionally, primary GBMs tend to occur in older patients, whereas secondary GBMs typically develop in younger patients.[9, 12, 16, 17] Histology can be used as a clue to determine whether a GBM is primary or secondary.[9, 12, 18] Overall, the average age at diagnosis is 64 years old, and it is 1.5 times more common in men than women.[9, 13, 19, 20] Incidence rates of GBM are higher in industrialized countries and are more commonly observed in whites.[9, 20, 21]

GBM Presentation

GBM has a variable presentation dependent mainly on its location. It can present with hemiparesis, signs of increased intracranial pressure, aphasia, seizures, memory loss, changes to personality, neuropsychological deficits, visual disturbance, ataxia, cranial nerve dysfunction, and/or dizziness.[16, 20] The great majority occur in the supratentorial cerebral cortex, with only 0.7% of GBMs occurring in the brain stem or cerebellum.[16] Approximately 44% of GBMs involve multiple lobes at the time of presentation, with unilobular GBMs most commonly occurring in the temporal lobe and in the frontal lobe.[13, 16] GBMs are typically characterized using MRI where they most commonly present as a ring-enhancing lesion with a hypointense, necrotic center on T1 MRI with contrast and as a hyperintense lesion surrounded by vasogenic edema on T2 MRI.[22, 23]

GBM Risk Factors

Most GBMs occur spontaneously. The cause and risk factors associated with the majority of GBMs diagnoses are unclear. Some risk factors that have been described are previous exposure to therapeutic ionizing radiation and to some environmental factors, e.g. vinyl chloride or pesticides, smoking, petroleum refining or production work, and employment in synthetic rubber manufacturing.[20, 24] GBM can also be associated with some rare genetic disorders, such as Turcot syndrome, Ollier disease, and Maffucci syndrome as well as the p53 mutation-related syndromes Li-Fraumeni syndrome and neurofibromatosis type 1.[20, 25] There has also been some association of GBM with certain viruses, such as cytomegalovirus.[26-29]

GBM Pathogenesis and Tumor Markers

The exact cellular origin of GBM is not definitively known.[30, 31] It has long been believed that GBM originates from glial cells. However, recent data has provided some evidence that neural stem cells, astrocytes, and oligodendrocyte precursor cells may each serve as the cells of origin.[30, 31] GBM tumors are differentiated from Grade 3 gliomas mainly by its characteristic areas of necrotic tissue surrounded by anaplastic cells and the presence of hyperplastic blood vessels.[32]

There are four subtypes of GBM that have been characterized: classical, neural, proneural, and mesenchymal.[33] Each of these subtypes is identified based on their varying levels of expression of various genes, molecules, and biomarkers, such as epidermal growth factor receptor (EGFR) and the gene TP53.[33, 34] An important marker used to determine prognosis and potential response to treatment is methylation of the promotor for the MGMT gene which encodes the DNA repair enzyme MGMT.[35, 36] The methylation of the promotor results in decreased expression of MGMT resulting in decreased capacity to repair damaged DNA in tumor cells and increased sensitivity to DNA-damaging therapeutics, such as temozolomide.[37]

GBM Treatment

GBM is extremely difficult to treat for several reasons. Because GBM tumor cells are highly invasive, it is nearly impossible to target a treatment at only the tumor without affecting the surrounding normal brain parenchyma.[38] Furthermore, GBM tumor cells are highly resistant to conventional therapies, such as chemotherapy and radiation, while normal brain cells are very susceptible to damage from these treatments and brain tissue has a limited capacity for

repair.[39] Finally, the blood-barrier makes it difficult for many therapies to reach all portions of the tumor.[39]

The current gold-standard treatment for newly diagnosed GBM is the Stupp protocol.[40] The protocol consists of a treatment combination of maximal safe surgical resection followed by radiotherapy and concomitant chemotherapy with temozolomide to the residual tumor.[40, 41] The treatment for recurrent GBM involves a combination of repeat surgery, radiotherapy, and/or chemotherapy.[19, 42]

There are several novel therapeutics being investigated as well.[42] These treatments range from alternative chemotherapeutics to gene therapy to immunotherapy to antiangiogenic agents.[42-44] Despite advances in surgical technique and medical science, novel treatments have been unable to prolong the survival of GBM patients.

GBM Prognosis

Currently, the survival of GBM patients receiving the Stupp protocol treatment is approximately 12-15 months, with less than 3-5% of patients surviving longer than 5 years.[40-42, 44] An increased age at the time of diagnosis carries a poorer prognosis with death typically occurring secondary to increased intracranial pressure from surrounding cerebral edema. The only currently measurable markers that have been found to be associated with increased survival are younger age at diagnosis, a greater Karnofsky Performance Score at diagnosis, MGMT methylation, and IDH1 gene mutation.[45, 46] In fact, MGMT methylation has been shown to increase a GBM tumor's susceptibility to the chemotherapeutic temozolomide used in the Stupp protocol.[47]

EMP2 Background

Epithelial membrane protein-2 (EMP2), also known as XMP, is a tetraspan protein that is part of the growth arrest-specific gene 3/peripheral myelin protein-22 (GAS3/PMP22) subfamily, which together with tetraspanins and connexins, comprise the three subfamilies of the large 4-transmembrane family.[3, 48] It was first described in 1996 by Taylor and Suter and by Ben-Porath and Benvenisty, separately.[49, 50] The gene is located on chromosome 16, and it transcribes a 167 amino acid membrane glycoprotein with four transmembrane domains (Figure 2).[48]

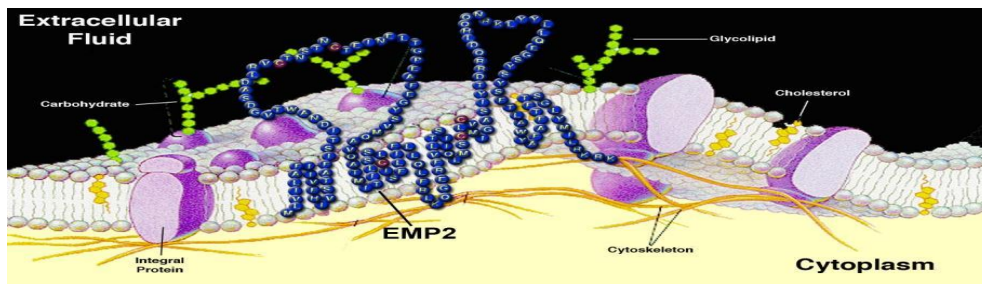


Figure 2. EMP2 structure. EMP2 is a 167 amino acid membrane glycoprotein with four transmembrane domains.

It has been shown by Wadehra et al. that EMP2 closely associates with the β_1 integrin subunit.[51] Within the cell, EMP2 has been found to localize to the plasma membrane, in addition to few other locations within the cell.[51] Specifically, it co-localizes with the $\alpha_6\beta_1$ integrin heterodimer, and its expression modulates the surface expression of $\alpha_6\beta_1$ integrin and the cell's binding to laminin, which is primarily mediated by $\alpha_6\beta_1$ integrin.[51] Further, EMP2 expression alters the integrin expression at the cell surface, specifically those with the α_5 or α_6 integrin subunits.[51] Therefore, EMP2 selectively associated with certain integrin isoforms, and EMP2 levels, in effect, regulate integrin surface expression and function.[51] Overall, it appears

that EMP2 levels modulate the selectivity of cell adhesion to extracellular membrane proteins, and this seems to result from EMP2-induced changes in $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrin surface expression.[51] It appears that the role that EMP2 plays in cell adhesion to extracellular proteins allows the cell to migrate through its extracellular matrix.[2]

EMP2 In GBM

EMP2 expression is significantly increased in human GBM cells compared to normal brain cells (Figure 3).[2, 8] Further, high EMP2 mRNA expression is an independent prognostic indicator of early death.[2] Western blot analysis also demonstrated increased expression in GBM whole tumor lysates and was detectable at various levels in all primary and established GBM cell lines.[2] In core samples from 110 patients, significant EMP2 expression was seen in the majority of malignant tissue compared to adjacent nonmalignant brain tissue with EMP2 being localized to both the membrane and within the cytoplasm.[2] When the expression was quantitated, the vast majority of tumor from GBM patients expressed some EMP2 compared with adjacent nonmalignant brain tissues.[2] EMP2 was also found to be a negative prognostic indicator, with higher levels predicting poor outcome (Figure 4).[2]

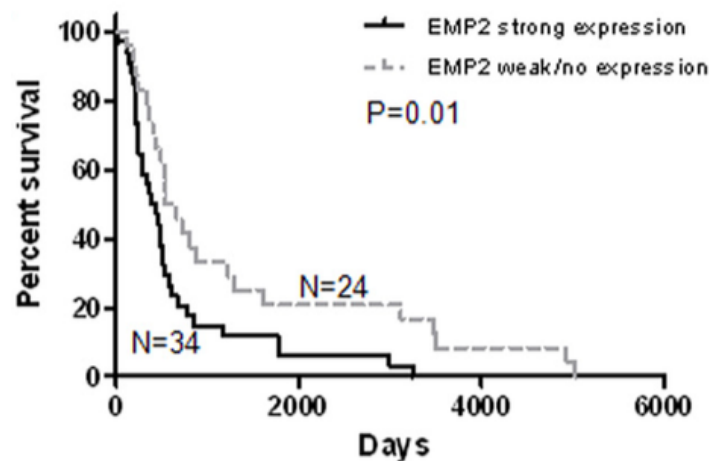
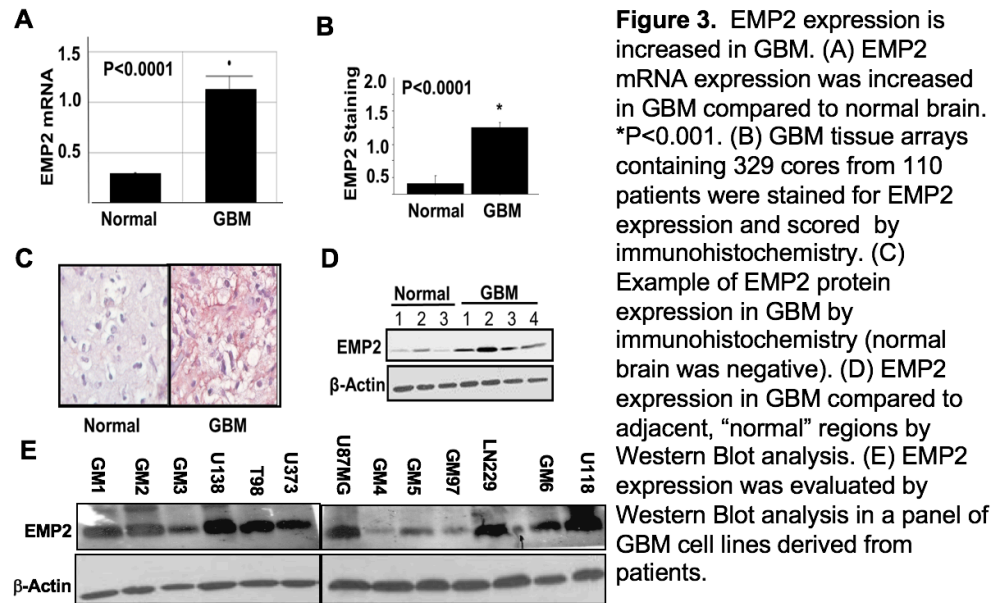


Figure 4. EMP2 expression decreases survival. EMP2 expression was dichotomized based on high (histological score, ≥ 2) or low (histological score, ≤ 1) expression. High EMP2 expression correlated with a poor survival.

Qin et al. also found that EMP2 accelerates GBM tumor growth rate by demonstrating that subcutaneous GBM growth in athymic nude mice was accelerated in cell lines

overexpressing EMP2 compared to those with a control vector.[2] Further, a reduction of EMP2 by either shRNA or ribozyme significantly decreased tumor size.[2] These findings suggest that EMP2 is an oncogenic protein that promotes tumorigenesis in GBM.[2]

EMP2 has been shown to up-regulate $\alpha v \beta 3$ integrin surface expression in endometrial cancer cells.[52] In GBM, EMP2 was found to significantly increase $\alpha v \beta 3$ integrin surface expression in all three cell lines studied; a significant reduction in EMP2 decreased $\alpha v \beta 3$ integrin surface expression in two of the three cell lines.[2] Integrins are known to transmit signals enhancing cancer proliferation and invasion.[53] Cell proliferation was not affected by overexpression or reduction in EMP2.[2] In one of the GBM cell lines, ectopic overexpression of EMP2 increased wound healing and cell migration compared to vector control-expressing cells, and a reduction of EMP2 expression decreased wound healing and cell migration.[2] Further, in two of the three cell lines, the increase in EMP-2 expression resulted in up-regulation of fibronectin-mediated cell invasion but had no effect on collagen-mediated cell invasion; $\alpha v \beta 3$ integrin adheres only to vitronectin and fibronectin but not collagen.[2] Thereby showing EMP-2 affects the cell's affinity for its ligand through its influence on $\alpha v \beta 3$ integrin surface expression. Additionally, specific antibodies against EMP2 or $\alpha v \beta 3$ integrin significantly impaired EMP2/integrin-mediated cell invasion and migration in a dose-dependent manner.[2]

Due its activation of $\alpha v \beta 3$ integrin activation, EMP2 alters cellular behavior through the recruitment of FAK and Src kinase.[54, 55] Similarly, EMP2 levels in GBM correlated with both FAK and Src activation through the increase or decrease in phosphorylation levels of each.[2] An increase in expression or knockdown of EMP2 increased or decreased, respectively, the level of phosphorylation of FAK and Src and, therefore, the activation of the integrin-FAK-Src signaling pathway.[2] These results were supported by immunohistochemical stains of patient

samples, which showed a positive correlation between EMP2 expression and Src phosphorylation.[2]

In vivo in athymic nude mice, EMP2 expression did not significantly increase tumor growth but it did lead to increased tumor invasion into the surrounding parenchyma, while a reduction in EMP2 significantly inhibited tumor growth compared to control mice.[2] These results suggest that targeting EMP2 expression may have a therapeutic benefit.[2]

Targeting EMP2 in Other Tumors

EMP2 expression has been shown to be up-regulated in a number of human cancers, including breast, ovarian, and endometrial malignancies. Furthermore, increased expression of EMP2 is a strong predictor of disease severity and progression in these cancers.[6] In vivo treatment of subcutaneous human xenografts of HEC-1A endometrial cancer cell lines with anti-EMP2 diabodies suppressed tumor growth and induced cell death.[56] These findings suggest that EMP2 may be a potential pharmacologic target for human endometrial cancer.[56]

Targeting EMP2 in breast cancer cells produced similar results. In in-vitro studies, anti-EMP2 diabodies and IgG1 both induced cell death via apoptosis of breast cancer cells lines and antibody-dependent cell cytotoxicity (ADCC).[4] In both xenograft and orthotopic mouse models, targeting breast cancer cells with anti-EMP2 diabodies or antibodies resulted in decreased tumor growth and increased tumor necrosis.[4]

EMP2 expression appears to promote cell migration and invasion via FAK and Src activation in breast cancer cell lines.[4] Furthermore, in-vitro experiments showed that anti-EMP2 diabody and IgG1 reduced cell migration and invasion through the significant reduction in Src phosphorylation.[4] In in-vivo models of metastatic breast cancer, systemic treatment with

anti-EMP2 IgG1 slowed tumor growth of the metastatic cells and increased necrosis throughout the metastatic tumor without causing any negative effects to the surrounding lung tissue.[4]

Similar to other cancers, targeting of EMP2 on ovarian cell lines with diabodies results in cytostasis and cell death via apoptosis.[5] In vivo, anti-EMP2 diabodies resulted in significantly reduced tumor load and increased necrosis.[5]

These results show that using antibodies to target EMP2 in tumors that express it can produce tumor regression and increased survival in preclinical studies. As such, we plan to show the same in preclinical studies for GBM tumors expressing EMP2.

Chapter 2

This chapter contains a proposal for a preclinical study similar to that found in a grant proposal. The purpose of the proposed study is to demonstrate the efficacy of targeting EMP2 on GBM tumor cells using an antibody against EMP2. A detailed description of the proposed study design is given followed by the dissemination and implementation potential of the results of this study.

Study Proposal

A. Objective

We hypothesize that the functional consequence of EMP2 up-regulation in GBM cells is to orchestrate the membrane expression of proteins required for cell migration, invasion, and neoangiogenesis. Additionally, we predict that the expression of EMP2 can be therapeutically exploited as a novel treatment target using antibodies against EMP2.

B. Rationale

Despite recent advances in molecular classification, surgery, radiotherapy, and the use of novel targeted therapies, the clinical outcome of patients with malignant brain tumors remains poor. The prognosis for patients with GBM, the most common and aggressive form of primary brain tumor, is extremely bleak with a median survival time of only 15 months. Hence, the development of a novel approach is critical to advancing the treatment of this disease. We propose to address this issue by studying a completely novel therapeutic target in GBM. Our compelling in-vitro and in-vivo studies, combined with the dramatic differences in expression levels of EMP2 in GBM versus normal brain, have led us to propose this project. This proposal focuses on understanding the role of EMP2 in GBM tumor formation as well as testing a novel

method to inhibit its expression. To achieve these goals, we have established a team of experts in EMP2 biology (Dr. Madhuri Wadehra) and in the clinical translation of GBM therapy (Dr. Isaac Yang) to supervise this project. In addition, we have collaborations with experts in EMP2 pathways (Dr. Lynn Gordon) and GBM pathology (Dr. William Yong) as we explore whether EMP2 can be exploited to achieve a novel therapeutic strategy using pre-clinical models.

C. Background

GBM is the most common and aggressive of primary brain tumor. Despite recent advances in molecular classification, surgery and radiotherapy, and the integration of novel molecular targeted therapies, the clinical outcome of patients with malignant brain tumors remains extremely poor. The current gold standard treatment of surgical resection, temozolomide chemotherapy, and radiation affords a median survival of less than 15 months.[57, 58] Continued investigation into the pathogenesis, biochemistry, and novel treatment strategies are prudent. The rapid and deadly course of the disease is due mainly to the highly invasive nature of the malignant cells into surrounding brain parenchyma making complete resection nearly impossible.[58]

It is believed that integrins play an important role in invasion by activating focal adhesion kinase (FAK), a nonreceptor cytoplasmic tyrosine kinase that is up-regulated in both anaplastic astrocytomas and GBM. FAK phosphorylation in turn activates Src kinase, which is part of the family of kinases that regulate the translation of extracellular signals with intracellular signaling. Dysregulated Src signaling has been demonstrated in many cancers, including GBM.

Epithelial membrane protein 2 (EMP2), a member of the growth arrest-specific gene 3/peripheral myelin protein-22 (GAS3/PMP22) group of tetraspanin proteins, has two

biochemical roles in receptor-mediated behavior. First, EMP2 regulates trafficking and intracellular compartmentalization and surface display of selected receptors and glycolipids by facilitating transfer of molecules from post-Golgi endosomal compartments to appropriate plasma membrane locations. Specifically, EMP2 appears to direct compartmentalization into glycolipids-enriched lipid raft microdomains.[59] Second, EMP2 physically associates with and regulates activity of integrin-FAK signaling complexes and has been shown to specifically associate with $\alpha v\beta 3$ integrin.[52] Interestingly, in GBM, $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins have been shown to correlate with disease progression and invasion into the brain parenchyma.[60, 61] These findings, concordant with the role of other members of the tetraspan family, indicate that EMP2 curates molecules at the cell surface that are brought into engagement with specific signaling complexes, and we have been able to show that EMP2 overexpression promotes a number of tumor-associated events including increased metastasis and invasion as well as increased angiogenesis through upregulation of HIF-1 α . [3]

EMP2 expression has been shown to be dysregulated in a number of human cancers. Examples include up-regulated in breast, ovarian, and endometrial malignancies; expression of EMP2 is typically a strong predictor of the severity of disease progression.[6] A similar situation is seen with EMP2 in primary brain cancer, where EMP2 is up-regulated at both the mRNA and protein levels in approximately 95% of patients as assessed by expression microarray and tissue microarray technology, respectively (see Preliminary Results). Significantly, to date, we have found no evidence of EMP2 expression in normal cells in the brain at either the RNA (PCR, microarray) or protein (immunohistochemistry, Western blot) level. As we will describe in detail below, the major goal of this proposed project is to more fully understand the function of EMP2

in GBM with an aim towards using this information as a strategic hub for a targeted molecular therapy.

Not much is known about EMP2's role in the central nervous system. A single recent study had shown that its mRNA is up-regulated in GBM, and its high expression was an independent prognostic indicator of early death.[8] The role of EMP2 as an oncogene in other cancers and its activation of FAK make it an intriguing target for further study. The study of EMP2 is of critical importance for understanding GBM invasion mechanism, development of targeted therapies, and assessment of treatment resistance. Inhibition of EMP2 has been tested in xenograft transplantation models; however no published studies using spontaneous brain tumor models are available to date. Furthermore, targeting EMP2 may have a beneficial and synergistic effect when combined with therapies that directly target malignant cells.

D. Preliminary Studies

EMP2 expression in GBMs but not healthy brain cells: Freije et al. found that EMP2 expression was increased 50 human GBM compared to 24 normal brain samples, and high EMP2 expression predicted early death (Figure 5).[8] This observation was further confirmed at the protein level by Western Blot analysis and immunohistochemistry (data not shown). EMP2 shows low/undetectable levels in the healthy brain using immunohistochemistry (IHC), PCR, or Western blot analysis. In addition to EMP2 being up-regulated in GBM, higher expression levels also proved to be an independent prognostic indicator for more aggressive variants of GBM (data not shown). We extended these results on a population basis using tissue microarray (TMA) technology. We screened 110 patients with primary brain malignancies. EMP2 protein levels were up-regulated in 95% of GBM patients compared to adjacent normal brain tissue (Figure

5B). In these samples, EMP2 was expressed on the plasma membrane of GBM cells but importantly, expression of EMP2 in normal brain was below the level of detection. Significantly, higher levels of EMP2 predicted an accelerated time to death compared with tumors with relatively lower levels (Figure 1C).

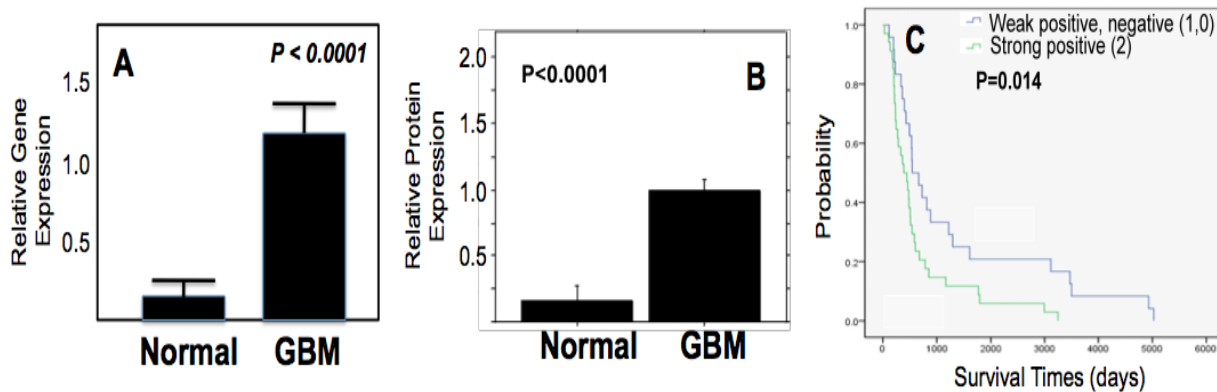


Figure 5. EMP2 expression in GBM and normal brain tissue. (A) EMP2 mRNA expression (Affymetrix microarray) was significantly increased in GBM tumor cells compared to normal brain tissue. (B) Using TMA(n=110 GBM; n=20 adjacent normal brain), EMP2 expression was significantly up-regulated in 95% of GBM patients at the protein level. (C) EMP2 expression was dichotomized into high and low expression. High EMP2 expression correlated with poor survival.

EMP2 is necessary for GBM tumorigenesis as its expression promotes tumor cell invasion and angiogenesis: Our central premise is that EMP2 is necessary for GBM tumorigenesis. As such, we tested the ability of GBM cells that overexpress EMP2, express a vector control, or express an EMP2 specific ribozyme (to decrease its expression) to form xenografts. Using a subcutaneous model, EMP2 over-expression produced larger and faster growing tumors than the vector control, while U373/Ribo failed to form any tumors even after 50 days (Figure 6A). To validate that this effect is reproducible in the brain, 5×10^6 Renilla luciferase-positive U-87 MG cells with endogenous (vector) or reduced levels of EMP2 (U87/shRNA) were created and

injected into the right forebrain. Dramatically, preliminary data suggest that U87/shRNA display a 75% reduction in tumor load over the vector control (Figure 6B,C).

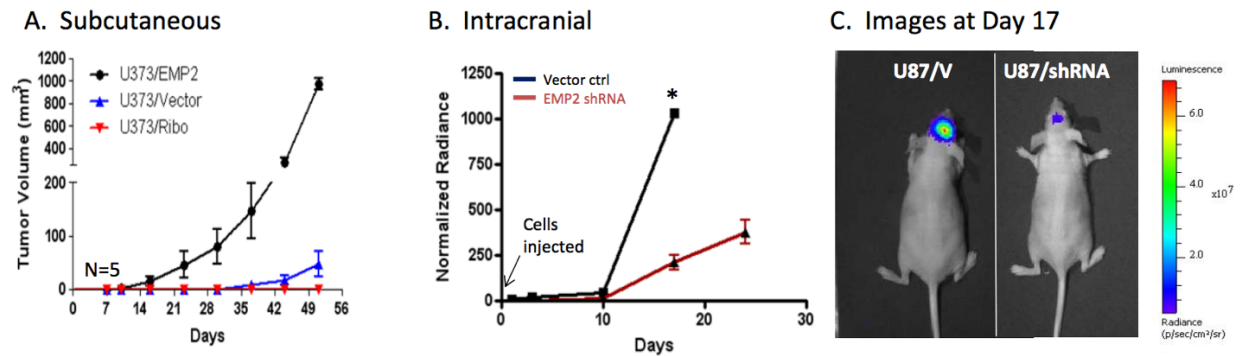


Figure 6. EMP2 expression promotes GBM tumorigenesis. (A) 2×10^6 U373/EMP2, vector control and knockdown (U373/Ribo) cells were injected subcutaneously. Tumor load was monitored for 50 days. N=6 (B) 5×10^6 Renilla luciferase positive U87/shRNA control or knockdown (EMP2 shRNA) were injected into the right forebrain. Tumor load was monitored over 24 days using bioluminescence imaging. Normalized radiance was calculated as maximum radiance minus minimum (flank) radiance. N=3. (C) Representative images of mice at day 17.

We have started elucidating the mechanism of EMP2 in GBM and have recently shown that EMP2 expression promotes FAK/Src activation. This change is relevant clinically as EMP2 expression and Src activation positively correlate in patients with GBM (Spearman's rank correlation coefficient=0.53; $P < 0.01$). We have started to characterize the downstream consequences for FAK/Src activation and can show that upregulation of EMP2 is sufficient to increase tumor cell invasion (Figure 7). Consistent with our observations, Fukushima et al. have shown that EMP2 mRNA correlates with GBM invasiveness.[62] In addition to invasion, another consequence of EMP2 expression is a change in neoangiogenesis. Upregulation of EMP2 was sufficient to induce HIF-1 α , HIF-1 β , and VEGF expression in multiple GBM cell lines (Figure 8A and 8B). This increase in VEGF expression was also sufficient to promote HUVEC capillary tube formation and an increase in HUVEC cell migration (Figure 8C, D), suggesting that EMP2

may be necessary for neoangiogenesis. Similarly, a reduction in EMP2 levels through either ribozyme or shRNA vectors abrogated these responses.

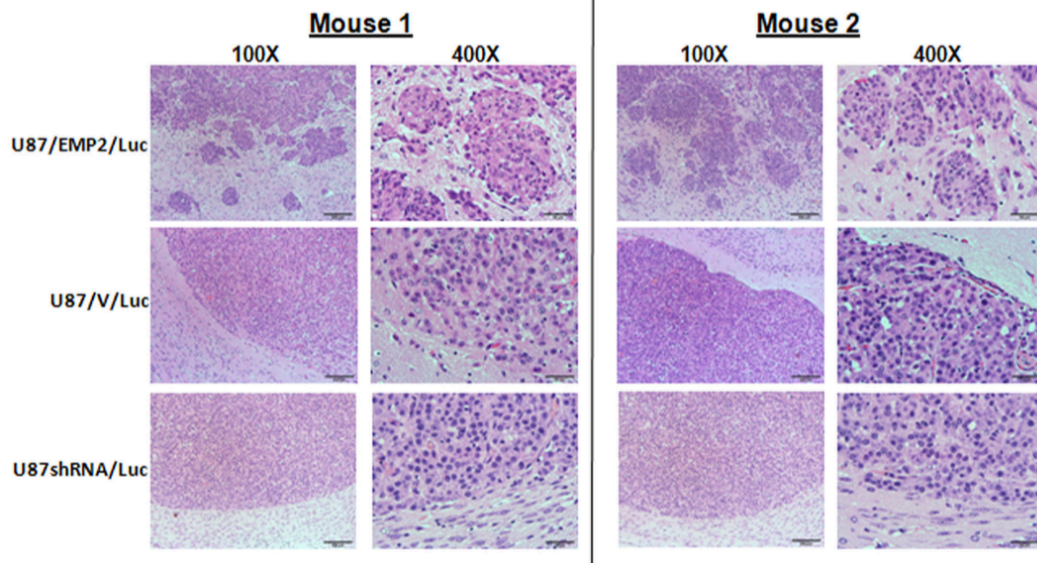


Figure 7. EMP2 expression promotes tumor migration *in-vivo*. Representative low and high magnification images from two U87/EMP2, U87/V, and U87/shRNA tumors. The high magnification image centers on the brain-tumor margin. Magnification: left, 100; right, 400.

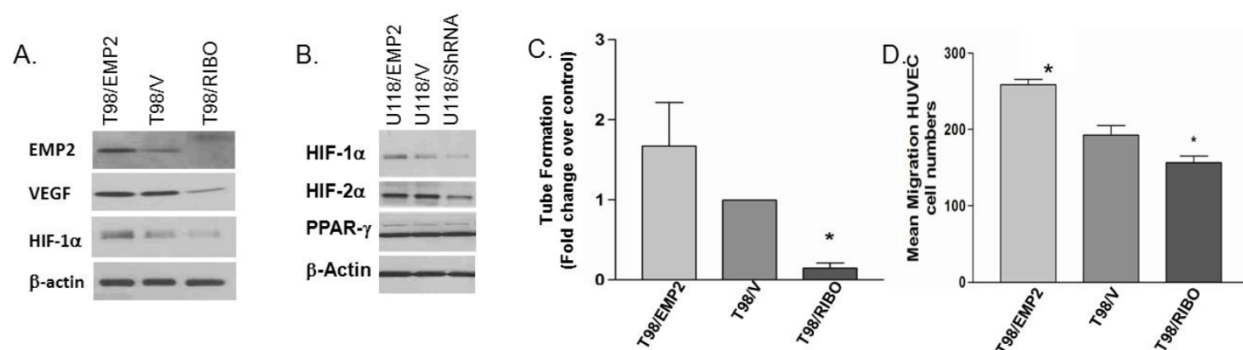


Figure 8. EMP2 expression promotes neoangiogenesis. (A) VEGF and HIF-1 expression correlates with EMP2 levels in hypoxic T98 GBM cells. (B) EMP2 expression was ectopically overexpressed (U118/EMP2) or knocked down using an shRNA vector (U118/shRNA). EMP2 levels alter HIF-1a and HIF-2a levels in U118 GBM cells. (C) Cultured supernatants from hypoxic T98 cells were added to HUVEC cells plated on low growth factor matrigel. Tubes were counted using light microscopy from three independent experiments. (D) HUVEC cell migration using a transwell migration assay.

EMP2 is a promising molecular target for GBM therapy: Because EMP2 was up-regulated in the majority of human GBMs examined to date, we evaluated whether EMP2 might be a viable target of attack in GBM. Previously, we constructed a number of recombinant immunoglobulin (Ig)-based reagents designed for use in EMP2 detection and targeting. These reagents include high-affinity diabodies (bivalent scFv dimers, 55 kDa) as well as a full-length IgG (150 kDa); all of these reagents bind to the extracellular loop of EMP2.[56] These Ig variants show cross-reactivity for mouse and human EMP2 as detected by flow cytometry and immunohistochemistry.[56] The rationale for constructing different sized immunoglobulins was to create a panel of reagents with properties tailored to *in-vitro* assays as well as tumor infiltration (*in-vivo* imaging and therapeutics) and clearance (i.e., optimized properties for either imaging or therapeutics). For this proposal, anti-EMP2 diabodies and full-length antibodies will be tested for targeting GBM.

We have already used these reagents to study a number of other cancers *in-vitro* and *in-vivo* such as human endometrial, breast, and ovarian malignancies. The results for these studies give us enthusiasm and optimism for our strategy regarding GBM and, importantly, a conceptual framework with which to move forward. Importantly, to date, healthy mice administered with excess anti-EMP2 reagents systemically (up to 80mg/kg/week) have shown no animal toxicity as assessed by overall health, weight, serum indicators of metabolic function and liver status, CBC counts, and tissue histology.[56] To begin to assess the efficacy of anti-EMP2 treatment as a therapy for GBM, we initially treated tumors with molar equivalents of the EMP2 diabody or IgG1. Both anti-EMP2 agents were able to induce apoptosis in both primary as well as GBM tumor cell lines (Figure 9A). To translate this effect *in-vivo*, as a proof of concept, we have treated rapidly growing U87MG/EGFR VIII GBM tumors implanted subcutaneously with intra-

tumor injections of the diabody. Anti-EMP2 diabodies reduced tumor load in this rapidly growing tumor (Figure 9B). We next tested the efficacy of anti-EMP2 IgG1 to reduce GBM tumor load. As the functional affinity of the EMP2 IgG1 is 0.1 nM, we tested the ability of the antibody to inhibit GBM tumor growth systemically. 3mg/kg injections twice per week of the EMP2 IgG1 significantly reduced growth in the U373 tumor model (Figure 9C).

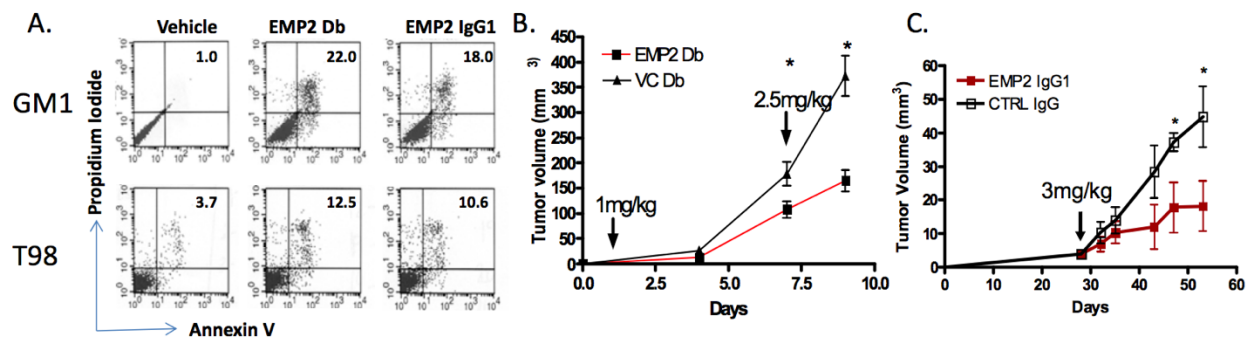


Figure 9. EMP2 antibody treatment induces GBM cell death. (A) A primary GBM tumor cell line obtained from Paul Mischel (GM1) or the T98 cell line was incubated with molar equivalents of the EMP2 diabody (Db) or the EMP2 IgG1. Cell death was measured using propidium iodide and annexin V staining. (B) The aggressive U87MG/EGFRVIII tumor cell line was injected into Nude mice. Mice were treated when tumors reached 4 mm³ with 1mg/kg twice per week followed by 2.5 mg/kg twice per week of EMP2 Db or a vehicle control diabody (VC Db) N=6*, P<0.05. (C) U373 GBM cells were injected. When tumors reached between 6-9 mm³, mice were treated twice per week with 3 mg/kg of EMP2 IgG1 or a control antibody. N=6*, P<0.05.

This grant proposal offers the promise of a fresh approach to GBM therapy. Our ambitious goal is to capitalize on the expression pattern and biological activity of membrane protein EMP2 as a viable target for GBM killing. Functionally, EMP2 plays an important role in integrin expression and FAK activation and in a more invasive phenotype. It also appears to promote neoangiogenesis, suggesting that its expression is important for efficient tumor formation. Given that we have compelling evidence *in-vitro* and *in-vivo* for other malignancies and in subcutaneous GBM xenografts, we predict that targeting EMP2 may provide a novel

therapeutic option. Of further note is the dichotomy of expression of EMP2 in GBM, as the protein is highly expressed in 95% of human GBM examined, yet has not been detected to date in any normal component of the brain. Therefore, we are encouraged and optimistic that through these experiments we will identify a target in GBM that alone, or in combination with other therapies, may have a dramatic clinical impact.

E. Research Design and Methods:

Aim 1: Characterization of EMP2 expression and GBM tumorigenesis using *in-vivo* models.

Rationale: Preliminary data suggest that EMP2 is an important contributor to GBM tumorigenicity in a subcutaneous mouse model. However, the role of EMP2 in GBM formation in the natural environment has not been examined. It is hypothesized that EMP2 expression is critical for GBM pathogenesis and that the functional consequence of EMP2 up-regulation in GBM cells is to orchestrate the membrane expression of proteins required for cell migration, invasion, and neoangiogenesis. Preliminary results from Dr. Wadehra's lab suggest - both in GBM and other systems - that EMP2 through modulating integrin surface expression activates the FAK signaling branch affecting cellular adhesion and migration.[3, 63, 64] Of note, Dr. Wadehra has explored the activation of FAK/Src kinase in GBM by EMP2, and hence, the downstream consequences of this activation focusing on the HIF proteins will be explored in this Aim.

Moreover, in this Aim, whether EMP2 expression levels correlate with tumor formation will be examined using an intracranial mouse model system. Tumor load and tumor cell invasion will be monitored using a combination of live animal imaging as well as conventional serial

immunohistochemistry. In addition, in line with the overall programmatic goal of identifying molecular tools for monitoring and attacking GBM, the molecular and phenotypic consequences of EMP2 upregulation with regard to neoangiogenesis *in-vivo* as well as in patients will be characterized. The focus will be on the regulation of hypoxia inducible factors as they have been known to regulate VEGF expression, glycolysis, and cancer stem cell formation.[62, 65-67] A more complete understanding of EMP2 up-regulation in GBM *in-vivo* will guide the rationale of targeting EMP2 and other pathway proteins as biomarkers for detection, prediction, visualization, and/or therapeutic attack.

Experimental Approach:

1.1 Investigate the mechanism of EMP2 control of HIF proteins using in-vitro models

It is proposed that EMP2 promotes HIF protein expression via activation of FAK and Src. It will be determined if this signaling pathway is necessary for HIF protein expression. In this section, experiments will address three issues: (a) the hypothesis that EMP2 activates HIF expression through FAK and Src activation; (b) the effects of anti-EMP2 antibodies as well as FAK and Src inhibitors on regulating HIF protein expression; and (c) the hypothesis that EMP2 control of HIF expression promotes cancer stem cells. These experiments will be performed on both primary and GBM cell lines.

Experimentation: Primary as well as GBM cell lines will be transiently transfected with EMP2, FAK, or Src small interfering RNA (siRNA) (commercially available from ThermoScientific) and placed in a hypoxic chamber (0.5%) for varying times.[68] Cells will be probed for HIF-1, HIF-2, Src, FAK, and EMP2 expression. To further validate these experiments, GBM cells will be further treated with pathway specific drugs (e.g. anti-EMP2

IgG1 for EMP2; dasatinib to target tyrosine kinases; the small molecule PP2 to target FAK-Src signaling and PP3 as a negative control).

The second set of experiments will utilize a number of GBM cell lines in which EMP2 expression has been modulated. The Wadehra lab has designed cells that ectopically overexpress EMP2 as well as reduce its expression via shRNA lentiviral infection or the use of ribozymes.[52] Panels have been established in well-characterized cell lines including T98, U87MG, U118, and U373, and these cells have been infected with firefly luciferase to allow for live animal imaging.[69, 70] Of note, some cells will be grown in defined serum free culture conditions where appropriate.[71] Cell lysates will be probed for the proteins above as well as other markers for GBM cancer stem cells (CD133, Notch, Glut 1, Oct4).[65, 72, 73]

Expected Outcomes: Understanding how pervasive the regulation of HIF proteins by EMP2 will be important in deciphering its role in GBM pathogenesis. A better understanding of HIF protein control is of paramount importance in tumor biology given their role in the creation of cancer stem cells. It is widely believed that hypoxia-inducible factor 1 α and 2 α are critical for the induction of specific tumor stem cell signature genes, and this sub-aim will show if EMP2 expression is necessary for this induction.[72, 73] While it is possible that cancer stem cell expression is not dependent on EMP2, understanding the pathways necessary that require EMP2 will be important to deciphering its role in tumorigenesis.

1.2 Characterization of EMP2 and GBM tumorigenicity

Establishment of intracranial models: Panels with modified EMP2 expression have been established in well-characterized cell lines including T98, U87MG, U118, and U373, and these cells have been infected with firefly luciferase to allow for live animal imaging.[69, 70] Prior to

implantation, mice will be anesthetized with intraperitoneal injection of ketamine, xylazine, and buprenorphine, placed in a stereotactic apparatus and an incision will be made over the cranial midline with a scalpel. Tumor cells (10^6 - 10^7 cells/ml) will be infused over the course of 3 minutes into the right forebrain (anterior-posterior = + 1.0mm from the bregma; mediolateral = +1.5 mm from the midline; dorso-ventral = -3.0 mm) as this region is mostly striatum and shows minimal ventricular leakage. The burr hole will be closed with bone wax and the incision sutured. Dr. Wadehra has been trained in these procedures but additional expertise is available through the laboratories of our collaborator Dr. Yang.

Evaluation of tumorigenicity: Bioluminescent cells will be detectable from the day of implantation and the tumor can be analyzed using the IVIS Spectrum instrument. Animals will receive a 150µg luciferin/kg body weight subcutaneous injection and imaged after 20 minutes. Tumor burden will be quantified over time using the mean and maximum tumor bioluminescence. Tumor bearing mice will be observed daily to assess morbidity. Mice will be euthanized when significant morbidity, a 10% change in weight, or symptoms, such as failure to groom or remaining in a hunched position, are observed. At that time, the brain will be dissected and luciferin will be added topically to obtain an *ex-vivo* image. The brain will then be fixed in formalin, processed, and evaluated by the criteria outlined in Table 1.

Parameters to be Examined	Method of Analysis
Gross examination of tumor	Gross pathological examination
Tumor pathology (cell morphology, invasion, angiogenesis, immune influx)	H & E staining of paraffin sections; CD31 and fitc-lectin staining for vasculature
EMP2 expression	IHC
Cell death (apoptosis and/or necrosis)	H&E staining, Caspase activation, TUNEL staining
Angiogenic and Stem cell markers(31, 32)	IHC staining for CD133, ALDH, HIF-1 α , HIF-2 α

Table 1. Evaluation of GBM tumors

Expected Outcomes: Data from subcutaneous GBM tumors suggest that EMP2 promotes tumorigenicity, and it is predicted that a similar outcome will be observed using intracranial models. The combination of bioluminescence and conventional histology will allow for accurate quantitation and qualitative description of tumor load. Given that subcutaneous tumors show a significant difference in tumor forming potential based on EMP2 levels, it is anticipated that intracranial tumorigenicity will positively correlate with levels of EMP2 as well. However, it is possible that EMP2 will not alter GBM tumor load in one or any of the cell lines created. In the case of the former, this would be intriguing as it would suggest that cells addicted to specific pathways require EMP2 expression. In this case, a detailed characterization of signaling using phosphoproteomics or candidate western blots could be employed. In the case of the latter, even if EMP2 levels do not correlate with tumorigenicity, this would still not preclude its utility as a therapeutic or diagnostic marker.

1.3 Examine the association between EMP2 expression and VEGF signaling in human GBMs

Preliminary data from Dr. Wadehra's lab suggests that increased EMP2 levels - such as those seen in GBM - will alter the surface integrin repertoire and result in FAK/Src signal cascade activation. Concordantly, it has been observed that high EMP2 correlates with Src activation in human patients. Recently, Dr. Wadehra's group has shown that EMP2 expression correlates with changes in VEGF and neoangiogenesis and that EMP2 promotes HIF-1 α expression in endometrial cancer as well as in a GBM cell line.[68] Unlike endometrial tumors, GBM cells can express two different hypoxic regulated factors, HIF1 and HIF2. To expand our understanding of EMP2 signaling in GBM, we will determine if HIF-1 α or HIF-2 α levels are

regulated by EMP2. Both transcriptions are known to regulate VEGF expression and neoangiogenesis, and both have been implicated to different extents with glioma cancer stem cells and/or GBM tumor formation.[62, 67, 74] Differences in expression will be determined using cell lines with modified EMP2 expression as well as using a high-density human brain cancer TMA which was constructed at UCLA (Dr. Paul Mischel, collaborator). It should be noted that this TMA has been previously used for assessing EMP2 expression (see Preliminary Results). Dr. Lee Goodglick is a leader in biomarker discovery and characterization using the TMA platform and together with Dr. Mischel has extensive expertise and experience with staining, quantification of staining, and biostatistical analyses of single and multiple marker.[75-81]

Experimental Design: Standard western blots will be conducted on GBM cells with modified EMP2 levels. Cells will be tested under both normoxic and hypoxic conditions, probing for the expression of HIF-1 α and HIF-2 α using commercially available antibodies. In addition, additional targets of each factor, such as Glut-1 or CD133, will be probed.[65, 67, 82] The brain cancer TMA contains 110 patients with primary brain malignancies. The array's patient population and the TMA characteristics have previously been described.[83, 84] Each TMA spot is linked to clinical and outcome data using a custom-made Oracle 8i Relational Database Management System (RDBMS) handling greater than 700 data fields. Record management is IRB- and HIPAA-compliant with no individual identifiers used, and the patient codebook is managed external to the system. Clinical information includes age, gender, histology, grade, tumor size, medical history, date of diagnosis, and death due to disease.

Protein expression using the GBA TMA: The TMA will be double stained for EMP2 plus HIF-1 α or HIF- 2 α using standard immunohistochemical (IHC) protocols routinely used in our

laboratory. The 'Envision+' (DAKO) multi-step amplification system with 3,3'-Diaminobenzidine (DAB) for EMP2 staining will be used, and Vector NovaRed (Vector) for integrin or phospho-protein staining. Dr. Wadehra has an established track record in analyzing protein expression in a variety of tissues as well as staining of TMA. Therefore, no complications are anticipated. Appropriate controls will be included to assess specificity such as i) appropriate pre-immune controls; ii) dose-dependent titration; iii) known positive and negative tissues; and when available iv) peptide or protein competitive inhibitory reagents.

Scoring and Interpretation of IHC Staining: Care will be taken to evaluate staining of specific target structures only. Both the intensity of staining and the percentage and type of neoplastic or normal cells expressing antigen are recorded. For double staining, a score will be given for each antigen. Note, if two stains are too difficult to differentiate (and thus score), single staining on serial sections will be performed. A board-certified pathologist (Dr. William Yong) will supervise the scoring. Importantly, we have experience and a track record of success with regard to scoring TMAs.[75-78, 80, 85, 86] We understand the difficulty with quantitation of IHC staining. Therefore, if necessary, we are prepared to utilize the semi-automated Aperio ScanScope CS workstation in our laboratory to quantify expression per given area of relevant cells.

Biostatistical Analysis of TMA Data: Dr. Wadehra's group is one of the leaders worldwide in biostatistical analyses of TMA data. This analysis will be done under the expert guidance of Dr. Steve Horvath (Biostatistics).[75-78, 80, 85, 86] Briefly, the linked marker data is converted into Stata or R statistical analysis software format (Stata Corp, TX, version 7.0; <http://www.r-project.org/>). Expression values representative of multiple tissue spots per case are converted into singular case-specific expression values representative at the case level, utilizing

median pooling. The robustness of the results will be evaluated with regard to alternative pooling strategies (mean, max, min). The case-specific data will be associated with the numerous clinical and pathological variables contained within the patient database. Conventional (e.g., correlations and regressions) and advanced (e.g. tree structured and clustering algorithms) data analysis techniques are utilized. The default settings of the RPART function in R for survival tree analysis are used to find appropriate cut-offs for classifying patients according to amount of marker expression. To avoid bias due to cut-off selection, we also report p-values for the undichotomized variables and will use a p-value correction method described previously.[77, 87] The Kaplan-Meier (KM) method is used to estimate survival time distribution; log-rank analysis is applied to test the difference between survival functions. The Cox proportional hazards model is used to test the statistical independence and significance of marker expression in predicting the risk of patient death based on a variety of potential prognostic factors. Discriminatory power is measured using the concordance (C-) index which equals the area under the ROC curve when dealing with a binary outcome. The Mann-Whitney U test or Kruskal Wallis rank sum is used to determine statistical significance of marker expression in primary tumors compared to metastatic lesions where available. Markers are also correlated with each other (via Spearman correlation), and clustered to investigate potential inter-relationships. All P-values are two sided, and $P < 0.05$ is considered significant. To avoid over-fitting due to dichotomizing tumor marker expression, we will take care to use both internal and external validation methods. For example, internal validation of outcomes predictions, performed as described using 400 bootstrapped samples helps guarantee that data are not over-fit. Validation of the results will be addressed by breaking submitted data into ‘training’ and ‘validation’ sets. The external validation is based on using the resultant model on external data and/or assays done in-house on patient materials provided from

outside collaborators. We have access to such collaborators as necessary through the NCI-EDRN program.

Potential Outcomes: Our prediction is that EMP2 expression in GBM modulates HIF-1 α expression as this has been observed in endometrial cancer.[68] However, it is possible that it also differentially regulates HIF-2 α expression. We recognize that results from this sub-aim are just the beginning and require many additional questions to be addressed which are beyond the scope of this grant, e.g. which pathways are necessary for this activation and are other downstream proteins regulated by the HIFs altered by EMP2 expression as well. If it is true that EMP2 regulates the expression of hypoxic inducible factors, we should be able to map these changes in human tumors using TMA technology. Moreover, the TMA platform allows us the opportunity to correlate marker findings with clinical and pathological information. Unfortunately, since GBM is such an aggressive, deadly disease, any predicted survival advantage based on one or more protein marker expression/activation, may be slight.

Realistically, it is unlikely that all cells in all GBMs examined on the TMA will show the exact same profile. We suspect there will be intra- and inter-tumor variability. Therefore, we predict these studies will show us both general patterns and degrees of heterogeneity. This, of course, will be important when designing multi-pronged therapeutic approaches (outside the scope of this current proposal). Nevertheless, we remain optimistic that these studies will, at the very least, confirm general trends observed in vitro and in animal models, and supply important information for future "personalized" therapeutic strategies.

Aim 2: Determine the cellular effects of treating GBM cells with recombinant anti-EMP2 reagents *in-vivo*

Rationale: Recombinant antibody immunotherapy has emerged as a robust treatment strategy for a variety of malignancies, but this technology has lagged for the treatment of brain tumors. A major therapeutic challenge for the use of antibodies is their inability to cross the blood–brain barrier (BBB) to reach tumors localized in the CNS.[88] In Aim 2, we propose to capitalize on the important finding regarding anti-EMP2 antibody reagents as a potential therapeutic strategy for GBM. This approach is especially relevant given the fact that the majority of human GBM tumors up-regulate EMP2 surface expression. We will specifically focus on the anti-EMP2 diabody and full-length IgG to more fully characterize the effects of anti-EMP2 treatment using *in-vivo* imaging on intracranial models of GBM (Figure 10).

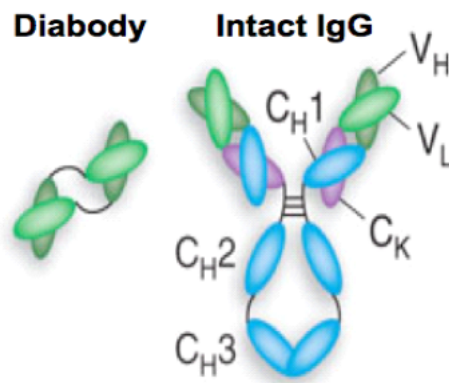


Figure 10. Molecular structure of a diabody and intact IgG1.

In Aim 2.1, we will test if direct injection of our anti-EMP2 IgG1 or diabody reduces tumor load as well as deduce any potential toxicity from their administration. Importantly, systemic administration of our EMP2 IgG1 up to 40mg/kg did not induce toxicity.[4] This aim will be performed on both existing GBM cell lines as well as primary tumor grafts. Recognizing that EMP2 treatment will likely be combined with existing therapies, Aim

2.2 tests if there can be a synergistic or addictive effect of dasatinib, temozolomide, and select chemotherapy drugs with anti-EMP2 agents.

Experimental Approach:

2.1 Direct Injection: The purpose of this sub-aim is to compare two anti-EMP2 recombinant antibodies (diabody and IgG1) in preclinical models of GBM. These antibodies will be tagged with firefly luciferase and tested for their ability to reduce tumor load as monitored by bioluminescence (described in Aim 1). While we recognize that this experiment will not address the ability of antibodies to cross the BBB, it will address two important questions: i) Is there any toxicity of the anti-EMP2 diabody and full length IgG in the brain? ii) Do these reagents when administered within the tumor inhibit growth and/or produce any survival benefit?

In this sub-aim, antibodies will be directly delivered into the brain of animal models by intracranial injection. Luciferase labeled U87MG tumor cells will be implanted as in Aim 1 as it known that anti-EMP2 antibodies reduced subcutaneous tumors created from this cell type. At the same time or on Day 7, anesthetized athymic mice will be placed in the stereotaxic apparatus and treated with 10-200 μ g antibodies using a 10 μ L volume administered at a rate of 0.5 μ L/min using the same coordinates as for tumor cell implantation. Antibodies will be administered weekly for up to 1 month through the same burrhole.

Both *in-vitro* primary cultures and orthotopic xenograft tumor models are crucial preclinical evaluation platforms for new anti-cancer therapeutics. Primary cell lines have been created by both Drs. Paul Mischel and Robert Prins and are available to us. Primary tumor grafts have been established by Dr. Robert Prins, and tissue is available on a recharge basis from the UCLA Translational Pathology Core Laboratory. In order to validate that anti-EMP2 antibodies

can impair primary tumor graft growth, acutely dissociated GBM cells will be injected as above into the brains of immunodeficient NOG mice.[89] Mice with the reduction of the total body weight (>20%) will be sacrificed, and brains will be processed for paraffin or frozen section.

In order to understand any changes in permeability and/or edema caused by anti-EMP2 antibodies, magnetic resonance images will be collected on some mice following treatment.[90] All MRI will be acquired on a 9.4 Tesla MRI scanner available on a recharge basis in the UCLA Clinical and Translational Science Institute facility (Bruker Biospin, Billerica, MA). Animals will be anesthetized with a 50:50 mixture of O₂ and medical air plus 1.5% isoflurane and placed prone in a stereotactic frame. T2-weighted rapid acquisition with relaxation enhancement (RARE) images will be acquired to assess the tumor volume. Tumor blood vessel permeability (K-trans) will be assessed from Dynamic Contrast Enhanced MRI. The signal intensity in the tumor region of interest will be analyzed using a MATLAB program, which models the tumor signal enhancement using the two-compartment model of Tofts et al. to extract the volume transfer constant (K-trans), the volume of the extravascular extracellular space, and the flux rate constant between the extravascular extracellular space and the blood plasma.[91]

Potential Outcomes: We predict that use of these multiple fragment formats with different pharmacokinetic and clearance properties will allow us to determine the optimum antibody format for direct use in the brain. We will initially evaluate two anti-EMP2 agents. The diabody is a 55kD antibody fragment consisting of the VL-VH domains fused together; it exhibits superior tissue penetration but a shorter half-life than the full-length antibody.[92] Both reagents bind the same epitope on EMP2 and have been shown to bind EMP2 *in-vivo*.[93] However, in the brain, intra-tumoral IgG moves slowly from the injection site due to its large molecular size,

making it difficult to reach distant regions of the tumor.[88] Thus, we predict that the diabody may be more efficacious.

2.2 Determine if there is an additive or synergistic effect of treating GBM cells *in-vitro* with anti-EMP2 recombinant reagents and Dasatinib or temozolomide:

Src is a particularly intriguing target for therapy in GBM as it has been strongly implicated in pathogenesis.[43]

Dasatinib is a potent small-molecule inhibitor of Src family tyrosine kinases that is currently being tested in phase II clinical trials for GBM.[43] Dasatinib also inhibits other tyrosine kinases and first gained notoriety for inhibition of bcr-abl in Imatinib-resistant chronic myelogenous leukemia patients.[94] Src activation is critical for both integrin signaling as well as pathways linked to proliferation, survival, cell-cell contact, and tumor-induced angiogenesis.[43] We will also test Temozolomide in conjunction with anti-EMP2 reagents. Temozolomide is a DNA alkylating agent, which, over the past 5 years, has become a routinely prescribed compound for individuals with newly diagnosed GBM.[95]

In the experiments described below, we will test the cytotoxic effect of anti-EMP2 recombinant antibodies combined with Dasatinib. The goal here is to lay the foundation for a multi-drug therapy model to potentially outpace acquired cell resistance. Importantly, we have access to Dasatinib through a Material Transfer Agreement with Bristol-Myers Squibb and Dr. Paul Mischel. Analogously, we will also test anti-EMP2 recombinant antibodies in combination with a drug currently used in clinic, Temozolomide, to test additive or synergistic effects. We will obtain Temozolomide from the Schering-Plough Research Institute (New Jersey).

The overall strategy is to treat GBM cells with varying doses of anti-EMP2 recombinant antibody together with varying doses of Dasatinib or Temozolomide. We will emphasize the

lower dose range of both Dasatinib and anti-EMP2 recombinant reagents. Initially, we will start with two GBM cell lines (see Aim 1) but expand the experiment to include primary GBM cell lines. These primary cell lines will be provided by our collaborator Dr. Isaac Yang. Similarly, we will initially use the anti-EMP2 diabody, but subsequently, we will use the full length IgG as well. The initial dose range for Dasatinib (0-100 $\mu\text{g/ml}$) is extrapolated from the study by Lu, et al., at UCLA.[84] Temozolomide will be initially diluted in dimethyl sulfoxide before preparing a working stock diluted in serum-free media. We will start with a dose range of 0-500 μM as previously reported.[96]

For each experiment, the following outcomes and parameters will be measured at each time point and dose: i) cell proliferation and cell viability (cell counts and trypan blue exclusion); ii) apoptosis (Western blot of cleaved caspase 3 and poly (ADP-ribose) polymerase); iii) FAK activation and total expression (Western blot of total Src and p-FAK); iv) Src activation and total expression (Western blot of total Src and p-Src); and v) EMP2 expression.

Potential Outcomes: Anti-EMP2, Temozolomide, and Dasatinib have all been shown to be cytotoxic in various models of *in-vitro* or *in-vivo* tumor progression. Certainly, Dasatinib treatment has dramatic effects on GBM cells in the preclinical models which have been used. Temozolomide has found to be effective in the clinic. Therefore, it will be fascinating to see whether anti-EMP2 antibodies plus Dasatinib has an additive or synergistic effect. We predict it will, but the proof will be in the experiments. We decided to examine Dasatinib first primarily because of logistic reasons (the drug was being used in preclinical and clinical trials at UCLA) and due to compelling mechanistic reasons (i.e., targeting the critical Src pathway). Nevertheless, if no additive or synergistic effect is observed, there are additional compounds that we would consider in combination with EMP2. This could include traditional chemotherapeutic

compounds (e.g., carmustine) or more experimental compounds (anti-integrin $\alpha v\beta 3$ and $\alpha v\beta 5$ antibodies).

Future directions, innovation, and impact: Our results suggest that EMP2 may be a promising molecular therapeutic target in GBM, and additional work will be needed to determine whether anti-EMP2 antibodies can be combined with standard chemotherapy or molecularly-targeted treatments. Although it may be difficult to overcome the permeability issues in the brain using an antibody or antibody fragments, studies here indicate that EMP2 has an important role in GBM tumorigenesis and tumor progression. Moreover, a reduction of EMP2 in GBM cells was sufficient to inhibit tumor growth *in-vivo*, lending support to the idea that anti-EMP2 treatment may be therapeutically beneficial.

Conclusion

The purpose of this thesis is to develop a research proposal for a study characterizing the expression and role of EMP2 in GBM and the feasibility of targeting EMP2 on GBM cells using antibodies as a potential treatment. This research realm is of tremendous important to the GBM and tumor field because of the burden that the disease brings to its patients and their families, and the relatively few and temporary treatment options that are available. The first half of this thesis gives an overview of GBM and the current treatment regimens as well as the background of EMP2 and how its expression is targeted in other tumors. The second half of the thesis is the study proposal as was submitted to the Howard Hughes Medical Institute for funding.

Future Directions

There is still a long way to go in proving EMP2's role in GBM tumorigenesis and in proving that it can be targeted to treat GBM in patients. If this proposed preclinical study were to be successful, the next steps would be to run the study in larger mammals and then to prove that the antibodies are safe to be injected into humans and that they can be used to effectively treat GBMs in human patients. An Investigational New Drug application would be filed with the Food and Drug Administration, and if approved, Phase 1, 2, and 3 clinical trials would be conducted. These studies would lay the groundwork for developing EMP2 as a prognostic indicator for GBM response to treatment and outcome.

Personal Knowledge Gained

I have learned a great deal through the process of writing this thesis. I had the opportunity to review and learn about GBM, GBM markers, and GBM treatment as well as about EMP2 and

its role in tumorigenicity in other tumors and in GBM. Secondly, I learned the process of developing and writing a research proposal for grant funding, a skill that will be valuable in my future career in academia. Finally, I have learned that there is much work to be done in finding a cure to GBM, and the ultimate cure will likely come from the amalgam of the many studies that are investigating the various treatment targets of GBM.

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