8-2016

The Overexpression of Basigin-3 in Glioblastoma

Samantha M. Wightman
Northern Michigan University, smw152@case.edu

THE OVEREXPRESSION OF BASIGIN-3 IN GLIOBLASTOMA

BY

Samantha Wightman

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

Master of Science

Office of Graduate Education and Research

August 2016

SIGNATURE APPROVAL FORM

The Overexpression of Basigin-3 in Glioblastoma

This thesis by Samantha Wightman is recommended for approval by the student's Thesis Committee and Department Head in the Department of Biology and by the Assistant Provost of Graduate Education and Research.

_____

Committee Chair: Robert Belton Jr, Ph.D Date

_____

First Reader: Robert Winn, Ph.D Date

_____

Second Reader: John Lawrence, Ph.D Date

_____

Department Head: John Rebers, Ph.D Date

_____

Dr. Robert Winn, Ph.D Date
Assistant Provost of Graduate Education and Research

ABSTRACT

THE OVEREXPRESSION OF BASIGIN-3 IN GLIOBLASTOMA

By
Samantha Wightman

Glioblastoma (GBM) is one of the most aggressive forms of brain tumor. With the current standard of care, survival prognosis for GBM patients is 15 months with a five-year survival rate of less than 3%. An increased understanding of the molecular mechanisms leading to cell growth and survival of GBMs may result in novel treatments to target and eradicate the disease. The protein Basigin-2 (aka EMMPRIN) induces the expression of matrix metalloproteinase (MMP) enzymes, and its expression level is positively correlated with GBM tumor grade. In 2011, Liao et al. reported that a splice variant of the basigin gene, called Basigin-3, may have an inhibitory function when bound to Basigin-2 in Human Hepatocellular Carcinoma (HHC) cells, as it decreases tumor growth, invasion and MMP expression. The goal of this study is to determine the effects of Basigin-3 overexpression in GBM cell growth. For this a recombinant fusion protein consisting of Basigin-3 and the red fluorescent protein mKate2 was overexpressed in a GBM cell line (LN229). RT-PCR and RT-qPCR was used to measure MMP gene expression in Basigin-3/mKate2 expressing cells, and confocal microscopy used to confirm over-expression of Basigin-3 in the transfected populations. Our hypothesis that Basigin-3 overexpression would reduce MMP expression cells was not supported by our data suggesting that Basigin-3 in GBM does not as an inhibitor of Basigin-2 function in GBM cells.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDEXIES

LITERATURE REVIEW AND BACKGROUND

INTRODUCTION TO GLIOBLASTOMA

Glioblastoma (GBM) is the most aggressive and life threatening form of primary metastatic brain tumor in humans. GBMs makes up 17% of all brain tumors, 60-70% of all astrocytomas, and have an incidence of 6.04 per 100,000 people per year in the United States (Association, 2010). Even with the use of standard treatment, the median survival rate for GBM is only 12.5 to 15 months and the five-year survival rate is less than 3% (Shibahara et al., 2013; Stupp et al., 2009; Weller et al., 2009; Yang et al., 2013). Clearly, an improved understanding of the molecular mechanisms regulating cell growth and survival of GBMs is need and could lead to novel treatments used to target and eradicate the disease.

GBM tumors are classified as gliomas; tumors that arise from the supporting cells of the central nervous system. More specifically, a GBM is a Grade IV astrocytoma, a tumor arising from astrocytes (Association, 2010). Grade IV tumors are the most malignant and aggressive. This grading system was developed by the World Health Organization (WHO), and grade IV tumors are characterized by hypercellularity, pleomorphism (individual cells vary in shape and size), frequent mitosis, microvascular proliferation and necrotic tissues are found within the tumor (Coons, 1996). There are many factors that can lead to the development of a malignant glioma including environmental, genetic, and other physiological factors, such as prior exposure to therapeutic doses of ionizing radiation and genetic predispositions including Li-Fraumeni syndrome, Neurofibromatiosis type 1 and 2, and tuberous sclerosis (Juratli, Schackert, & Krex, 2013).

TREATMENT OF GBM

The standard treatment for GBM tumors is surgical resection, followed by combined radiation and chemotherapy with temozolomide. Even with this standard treatment nearly all GBMs recur after initial therapy. The median survival rate of a patient diagnosed with GBM is about 12.5 to 15 months with current standard of treatment (Weller et al., 2009). A high rate of recurrence and few effective treatments are to blame for the low five-year survival rate of less than 3% (Stupp et al., 2009).

Surgical resection effectively reduces the size of the tumor, however complete resection is not possible due to the invasive nature of the tumors. To maximize the extent of tumor resection, intraoperative brain mapping and MRI along with 5-aminolevulinic acid (5-ALA) derived tumor fluorescence for intraoperative identification of malignant glioma tissue is used in some countries (Juratli et al., 2013). 5-ALA is a natural substrate of the heme synthesis pathway in mammalian cells were it is metabolized to produce the intermediate compound called protoporphyrin-IX (PpIX). PpIX is normally converted to heme by inserting the ferrous iron through the ferrochelatase enzyme within the mitochondria. In malignant tissue the amount of ferrochelatase is decreased and causes the accumulation of PpIX in cancer cells (Duffner F., 2005). PpIX is a photoactive compound and its accumulation in tumor cells makes it possible to visualize the cancerous cells within the brain when exposed to violet-blue light. In this way, cancer cells can be visualized by their fluorescence allowing for more complete resection of the tumor. Patients are given 5-ALA orally to increase the amount of 5-ALA in their system. The use of the 5-ALA treatment during tumor resection is helpful in removing majority of the tumor.

Because GBMs are diffuse tumors this resection technique still may not remove all of the tumor cells and leaves the chance for tumor reoccurrence (Eljamel, Goodman, & Moseley, 2008).

Radiation therapy uses high-energy radiation to damage cellular DNA in order to induce cell death in rapidly growing cells. Radiation therapy has been shown to be effective in the treatment of GBMs and increases the median survival rate from five to nine months compared to surgery alone. Glioma tumors are made up of a heterogeneous mixture of cell types, with some cells being more resistant to radiation therapy than others. Thus, after the full dosage of radiation has been applied, some of the tumor may still be present (Juratli et al., 2013). Radiation therapy causes double strand DNA breaks. It is possible for these breaks to be repaired by non-homologous end joining and is prone to error. Unrepaired double strand breaks trigger apoptosis, explaining why radiation is used as a treatment to kill cancerous cells (Roos & Kaina, 2006).

Chemotherapy is also used to treat various types of cancer by damaging the DNA in rapidly reproducing cells. The use of chemotherapeutics to treat GBMs has been difficult. Systemic chemotherapy approaches generally do not work for GBMs because the chemotherapeutic agents cannot pass the blood-brain or blood-tumor barrier (Juratli et al., 2013). There are a few chemotherapy agents that can cross the blood brain barrier or can be administered during surgery. In addition, GBMs can accumulate mutations that allow them to be better repair DNA damage caused by this therapeutic technique (Lawrence J. E., 2015).

There are two groups of chemicals that are used for chemotherapy and can cross the blood-brain barrier: alkylating agents and nitrosources (Juratli et al., 2013). The most widely used chemotherapy agent to treat GBMs is temozolomide (TMZ), an alkylating agent that is taken orally and can pass the blood brain barrier (Juratli et al., 2013; Lawrence J. E., 2015). TMZ is a prodrug and is converted into the active drug, 5-(3-methyltriazen-1-yl)imidazo-4-carboximide (MTIC). MTIC methylates the DNA most commonly on the N7 position of guanine and N3 position of adenine. The O6 position of guanine can be methylated as well but is less common. The addition of these methyl groups are toxic to the cell and will induce apoptosis if not repaired (Lawrence J. E., 2015). Tumor resistance to alkylating agents is a clinical problem caused by an overexpression of DNA repair enzymes. Alkylating agents add chemical groups to DNA which inhibit the cell cycle and stops cell growth (Juratli et al., 2013). The MGMT protein repairs the DNA by removing the chemical group, for example this protein can remove the methyl groups added by MTIC repairing the altered amino acids making them no longer cytotoxic (Juratli et al., 2013; Lawrence J. E., 2015). If the chemical group is left on the DNA and not repaired by the MGMT protein, the chemotherapy lesion induces cell apoptosis (Juratli et al., 2013).

Nitrosources are used to induce the alkylation to inhibit DNA replication. Carmustine can cross the blood-brain barrier and it is also effective against malignant glioma cell lines *in vitro*. However, it is also very toxic which is why systemic treatment with carmustine is limited. Carmustine wafers are also a form of alkylating chemotherapy where the drug-impregnated wafers are placed directly into the tumor resection, usually placed during a second surgery after the original resection. Since the wafers are used in a

second surgery, this treatment is usually reserved for younger patients with resectable tumors (Juratli et al., 2013).

CANCER

Cancer is defined as a group of diseases that is characterized by unregulated cell growth, invasion, and spread of cells (Pecorino, 2012). This change in cell growth and invasion can be caused by an accumulation of mutations within the genome as a result of a genetic disorder passed down from parent to progeny, exposure to environmental carcinogens, or by random mutations that occur throughout a person's life. The most common mutations resulting in tumor formation are mutations to either proto-oncogenes or tumor suppressor genes. Proto-oncogenes have a normal role in controlling cell growth or differentiation. When proto-oncogenes experience mutations resulting in elevated activity of the gene product, they become 'oncogenes' which promote cell growth and the formation of cancer. Tumor suppressor genes code for genes that negatively regulate cell growth. When these genes are altered in tumor cells they are changed in a way that decreases or abolishes their function. For a tumor to develop it usually is a result of the combination of multiple mutations but there are a few specific mutations that increase the chance of developing a tumor (Medicine, 1992). Several examples of the types of mutations that can promote cancer formation are outlined on the following pages.

Li-Fraumeni syndrome 1 is a rare autosomal dominant disorder that is characterized by an early onset of tumors. The most prevalent mutation associated with this syndrome is to the p53 gene on chromosome 17p13.1 (Bachinski, 2005). The p53 gene codes for a tumor suppressor protein called p53 which acts as a transcription factor for a

large number of genes whose gene products function in cellular growth arrest, DNA repair, apoptosis regulation, and anti-angiogenic proteins. When the p53 gene is mutated, like in Li-Fraumeni syndrome, the p53 protein loses its ability to act as a transcription factor for these genes. Without the function of the p53 protein there is a loss in cell growth control leading to an overall increase in cell proliferation. Li-Fraumeni syndrome is not the only instance where p53 is mutated. The mutation can occur spontaneously and is commonly found in many types of cancers, including GBMs (Weinburg, 2007). In a review of the molecular alterations that occur within different GBMs Dunn et al. demonstrated that p53 was mutated in 42% of the tumor samples. In this work they used the Cancer Genome Atlas Research Network to explore somatically mutated genes in GBMs (Dunn et al., 2012).

GENETICS OF GBM

Cancer is the product of many mutations and epigenetic modifications that build on each other (Weinburg, 2007). There are many signaling pathways that are altered in GBMs, including the WNT, Ras, and PI3K pathways as described above (Brems, 2009; Ferner, 2007; Tee, Manning, Roux, Cantley, & Blenis, 2003). The modification of signaling pathways in GBMs makes the understanding and treatment of GBMs very difficult (Zhu et al., 2009). Like other cancers, GBMs result from various mutations that can alter gene expression within a cell. Some genes tend to be more commonly mutated than others, including the genes p53, NF1, PTEN, and EGFR (Dunn et al., 2012).

Neurofibromatosis is also an autosomal dominant disorder within the NF1 gene. This gene codes for the protein neurofibromin that functions as a tumor suppressor by inhibiting the Ras and WNT pathways. Neurofibromin is normally found in neuroglial cells

such as schwann cells. Patients afflicted with neurofibromatosis produce decreased amounts of neurofibromin, thus allowing uncontrolled cell growth and the formation of tumors within the nervous system (Brems, 2009; Ferner, 2007). In a review of the molecular basis of GBMs, an analysis of tumors from the cancer Genome Atlas Network, NF1 is one of the genes most commonly found mutated in GBMs at 21% of the tumors analyzed (Dunn et al., 2012).

Phosphatase and Tensin Homolog (PTEN) is a tumor suppressor gene that is found on chromosome 10p23 (S. I. P. Wang, J.; Li, J.; Bruce, J. N.; Cairns, P.; Sidransky, D.; and Parsons, R., 1997). A common mutation found in over 90% of primary GBMs is the hemizygous or homozygous deletion of the PTEN gene (Gont, 2013). PTEN is a phosphatase enzyme that converts the second messenger molecule phosphatidylinositol 3,4,5 triphosphate (PIP3) to its inactive precursor phosphatidylinositol 3,4 bisphosphate (PIP2). As stated previously, the phosphoinositol-3-kinase (PI3K) signaling pathway regulates cell growth and proliferation by stimulating the formation of PIP3 in response to cell stimuli. Because The PI3K and PTEN enzymes have opposing functions, with the loss of PTEN increasing the production of PIP3 by PI3K. This results in increased cell survival and cell growth within GBM tumors (Gont, 2013; Pezzolesi, Zbuk, Waite, & Eng, 2007).

Epithelial Growth Factor Receptor (EGFR) is located on chromosome 7p11.2 and is mutated in nearly 50% of all GBMs (Brennan et al., 2013; Dunn et al., 2012; K. Wang, Yamamoto, Chin, Werb, & Vu, 2004; Zhu et al., 2009). EGFR and its ligand have a variety of functions in normal cells that are altered in cancer. For example, the amplification of the EGFR gene resulting in multiple copies of the gene increases cell proliferation due to

increased expression of EGFR. Another common EGFR mutation is an in-frame deletion of exons 2-7. This deletion produces a protein product called EGFRvIII that functions as a ligand-independent receptor (Zhu et al., 2009). These GBM mutations initiate early tumor development, sustain tumor growth, promote tumor infiltration, and resistance to therapy (K. Wang et al., 2004; Zhu et al., 2009).

Epigenetic alterations can also affect gene expression leading to GBM formation (Weinburg, 2007). An important epigenetic factor for the treatment of GBMs is DNA methylation. DNA methylation is the addition of a methyl group to the cytosine base of double-stranded DNA. This covalent addition also recruits proteins that bind to the methyl-cytosine group to alter the structure of the DNA (Alberts, 2008). One example of this type of epigenetic alteration in GBMs is the methylation of the O^6-methylguanine-DNA-methyltransferase (MGMT) gene promoter region. MGMT promoter methylation prevents MGMT gene expression and thus blocks MGMT function. MGMT is a DNA repair protein that removes chemical adducts from the O^6 position on guanine, and in doing so targets the MGMT protein for degradation. The silencing of this gene by promoter methylation results in a loss of MGMT gene expression preventing MGMT-mediated DNA repair. Because the MGMT enzyme functions to remove chemical adducts from DNA in response to alkylating chemotherapeutics, the silencing of this gene by methylation can increases the effectiveness of current chemotherapy treatments (Rivera et al., 2010). This is a prime example of the kind of information generated by basic research that can be taken advantage of in the treatment of GBM tumors.

Cancer cells that accumulate mutations from various genetic or environmental factors can alter their local tumor microenvironment through the proteins they produce. In other words, the proteins that the tumor cells produce can affect surrounding cells to support tumor survival and growth. For example, cells can release proteins from the cell surface through microvesicles or the secretion of soluble proteins. Interestingly, cancer cells produce elevated levels of some proteins and can stimulate surrounding stromal (normal) cells to express additional factors to reduce environmental constraints allowing for proliferation and metastasis of the tumor (Biswas C., 1995; Tsai et al., 2013). One example of a protein that can stimulate surrounding stromal cells is the cell surface glycoprotein basigin-2 (also called EMMPRIN). High-grade astrocytomas overexpress this protein and the expression level positively correlates with WHO grades for astrocytomas suggesting that the overexpression of this protein plays a role in the tumors increased ability to survive (Tsai et al., 2013). Furthermore, the release of basigin-2 from tumor cells stimulates the expression of MMP enzymes in the surrounding normal tissue. MMPs catalyze the degradation of the extracellular matrix within tissues allowing for the growth and metastasis of tumors. Developing an understanding of the mechanisms and molecules mediating tumor-stromal cell interactions is important in the development of treatments and diagnostic strategies for metastatic cancers(Belton, Chen, Mesquita, & Nowak, 2008).

BASIGIN GENE

The basigin gene produces four variants that belong to the immunoglobulin superfamily of cell surface glycoproteins. Basigin is a pleiotrophic molecule which means that it is under control of a single gene but has several distinct and unrelated effects on the cell's phenotype. Basigin plays critical roles in spermatogenesis, lymphocyte activation,

and expression of monocarboxlyate transporters, however basigin's best characterized function is its ability stimulate the expression of matrix metalloproteinases (MMPs) in stromal cells (Belton et al., 2008; Iacono, Brown, Greene, & Saouaf, 2007; Weidle, 2010). Basigin is known by other names such as Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), M6 Leukocyte Activation Antigen (M6) and CD147 Antigen (CD147) (McKusick and Converse). The basigin gene is found on Chromosome 19, the specific cytogenic location is 19p13.3 and the gene contains ten exons (McKusick and Converse).

There are four basigin isoforms (basigin-1, -2, -3, and -4) that possess a shared cytoplasmic and transmembrane domain but vary in their extracellular domains (Belton et al., 2008; Iacono et al., 2007). The best known and most common basigin protein is isoform 2 (basigin-2 or EMMPRIN). The transcriptional regulation, expression profiles and functions that distinguish the isoforms from each other are not well known. The basigin-1 transcript is found exclusively in the retina and is distinguished by possessing three immunoglobulin (Ig)-like domains in the extracellular portion of the protein. Basigin-2 is the predominant variant and is distinguished by having two Ig-like domains. Basigin-3 is a short isoform containing one Ig-like domain that interacts with the internalized basigin-2 during receptor-mediated endocytosis in an unknown manner (Belton et al., 2008). Little is known about the function of isoforms-3 or -4, but studies using hepatocarcinoma cells suggest that basigin-3 might function as an inhibitor of basigin-2(Liao et al., 2011). The basigin gene is widely expressed in normal cells, although there has not been extensive studies on the functions of basigin isoform 3 and 4, there have been many functions associated with basigin-2. Basigin-2 is the most highly expressed isoform and has been shown to be involved in different cellular mechanisms such as inducing the expression of

MMP, and the recruitment of monocarboxylic acid transporters (MCTs) and ABC transporters to the cell membrane (Bleau, Huse, & Holland, 2014; T., 2003).

Matrix malloproteinases are enzymes that break down elements of the extracellular matrix such as collagen, laminin, and fibronectin. Matrix degradation enzymes are activated by three different control mechanisms: local activation, confinement by cell-surface receptors, and secretion of inhibitors. Local activation is the mechanism by which proteases are secreted as inactive precursors that can be activated locally when needed. This activation method is used for more specific MMPs. Some MMPs are less specific and are more likely to be activated by the confinement of cell-surface receptors. This mechanism confines the enzymes to more specific areas where it is needed (Alberts, 2008).

There are four MCT isoforms (MCT1-4) and they have been shown to catalyze the transport of monocarboxylates (lactate, pyruvate, acetoacetate) across the cell membrane (Kirk P., 2000). The transport of monocarboxylates is very important for cell metabolism, especially the transport of lactic acid, an acidic byproduct of glycolysis, out of the cell (Kirk P., 2000; Le Floch et al., 2011). Glycolysis occurs in all cells and is most commonly used in hypoxic situations. Basigin-2 has been shown to be a chaperone for MCT1 and MCT4 to the cell membrane and is necessary for the function of these MCTs (Kirk P., 2000; Le Floch et al., 2011).

ABCG2 is a half-transporter of the G subfamily of ATP binding cassette (ABC) transporters (Bleau et al., 2014; Jin et al., 2009; Zhou et al., 2013). ABC transporters use ATP to transport small molecules across the membrane. This movement helps detoxify the cell of cytotoxic agents, including drugs used to induce cell death (Bleau et al., 2014). In

order for ABCG2 to be active it needs to form dimers or oligomers, which is where basigin-2 comes in. There is evidence that basigin-2 co-localizes with ABCG2 and can regulate ABCG2's dimerization this allows the transporter to become active (Zhou et al., 2013). ABCG2 is upregulated in many different tumor types and its ability to transport drugs out of the cell causes the cell to develop a multidrug resistance (Jin et al., 2009).

BASIGIN FUNCTION IN CANCER

MMPs are found in abundance at the boundary between the tumor and the surrounding stromal tissue. The stromal cells are stimulated to express and secrete MMPs into the tumor microenvironment by the tumor cells (Weinberg, 2007). The most abundant tumor-related MMPs are interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), and gelatinase A (MMP-2) (Sameshima, 2000).

Analysis of numerous tumors has revealed that the elevated expression of basigin in tumor cells is correlated with an increasingly aggressive tumor phenotype (lacono et al., 2007). This overexpression of basigin-2 sets in motion an increase in many of basigin-2's functions in cell survival. An increase in basigin-2 has also shown an increase in MCT expression. The tumor microenvironment is hypoxic due to the rapid cell growth. The lack of available oxygen for the rapidly growing tumor cells cause them to rely on fermentative glycolysis to survive. Lactic acid is byproduct of this type of metabolism but the accumulation within the cell is toxic. The increase of MCTs raises the amount of lactic acid the cell can transport across the membrane increasing the cell's chance of survival (Kirk P., 2000; Le Floch et al., 2011).

Cancer cells overexpress basigin-2 and shed microvesicles containing basigin proteins. This increase of basigin-2 increases the stimulation of MMPs and decreases physiological inhibitors in the extracellular matrix, allowing the tumor to metastasize (Belton et al., 2008; Iacono et al., 2007). Basigin-2 forms a homo-oliomer, a prerequisite to become fully functional, to stimulate MMPs secretion from surrounding stromal cells (Liao et al., 2011). Basigin-3 has also shown increased expression in cancer cells but is not expressed as highly as basigin-2. In a study done by Liao et al. involving human hepatocellular carcinoma, cells that overexpressed basigin-2 grew at the highest rates where cells that over-expressed basigin-3 grew at the lowest. These results suggest that basigin-2 promotes proliferation in cancer cells while basigin-3 inhibits proliferation. In human hepatocellular carcinoma lines basigin-3 interacts with basigin-2 to form a hetero-oligomer. This interaction may regulate basigin-2 stimulation of MMP (Liao et al., 2011).

SUMMARY

The mechanisms used by cancer cells to proliferate and metastasize are a result of accumulated genetic changes within the cancer cells and are not yet fully understood. Mutations in cancerous cells contribute to the production of abnormal amounts or functionally altered proteins which can change how the cell functions and interacts with its surrounding environment. Members of the basigin gene isoforms have be found to be overexpressed in different types of tumors, with basigin-2 (EMMPRIN) overexpression strongly correlated with tumor grade. One of its more studied functions is the ability to induce the expression and secretion of MMPs from surrounding tissues, promoting the ability of the tumor to grow and metastasize. The function of basigin-3 is not well known but there is evidence supporting that it plays a role in competitively binding to Basigin-2

inhibiting basigin-2's role in stimulating MMP secretion and decreases proliferation. Studying the localization of basigin-3 expression within glioblastoma (GBM) tumors and the changes in expression of proteins associated with basigin-2 stimulation could lead into more extensive studies of its functions and influences within cancer cells.

OBJECTIVES AND HYPOTHESES

The objective of this project was to study the effects of basigin-3 overexpression in GBM cell lines. The function of basigin-3 is not known, but there is evidence supporting its role in competitively binding to Basigin-2 and inhibiting basigin-2's ability to stimulate MMP expression. Measuring the effects of basigin-3 overexpression within glioblastoma (GBM) cell lines would lead into more extensive studies of its functions and influences within cancer cells.

Hypothesis: Basigin-3 overexpression in GBM cell lines will reduce MMP gene expression levels.

Aim 1: Generate stably-transfected GBM cell lines expressing a basigin-3/mKate2 fusion protein.

Aim 2: Demonstrate basigin-3 overexpression in GBM cell lines

Aim 3: Measure changes in MMP1, MMP2, MMP3 expression in cells overexpressing basigin-3.

METHODS

PLASMID AMPLIFICATION

All plasmids were purchased from Origene Technologies (Rockville, MD). The plasmids were transformed into DH5alpha E. coli competent cells according to standard procedures. The basigin-3, basigin-2, and mKate2 expression vectors were selected with ampicillin, and single bacterial colonies from each plate were selected and used to inoculate 5mL of LB broth for amplification. Subsequently, the 5mL cultures were used to inoculate 100mL LB broth cultures for large scale amplification. All cultures were grown in LB broth with ampicillin at 37°C on a shaker table at 200 RPM. Plasmids were purified from the bacterial cultures using Qiagen Plasmid mini and maxi kits (Valencia, CA). After purification, DNA concentration and purity were determined by UV spectrometry using a Nanodrop spectrophotometer (Thermo Fisher, Waltham MA). Restriction enzymes and agarose gel electrophoresis were used to verify plasmid identity. The restriction enzymes used for analysis of the plasmids were Mlu1 and AsisI.

SUBCLONING OF BASIGIN-3 INTO MKATE2 EXPRESSION VECTOR

The open reading frame (ORF) of basigin-3 was amplified using the high fidelity Phusion DNA polymerase purchased from ThermoFisher (Waltham MA). Primers were designed to generate novel restriction sites on the amplicon in order to preferentially clone the genes in correct orientation. Primer information can be found in Table 1. Once the gene inserts were amplified by PCR, the ends were digested with the appropriate restriction enzymes. The gene inserts were verified and purified by agarose gel electrophoresis. The digested PCR product band was excised from the agrose gel and purified using QIAquick gel

extraction kit from Qiagen (Valencia, CA). The mKate2 expression vector was digested using Mlu1 and Asis1 and was also purified through gel electrophoresis. The basigin-3 ORF was ligated into the mKate2 expression vector using New England BioLab's Quick Ligation Kit (Cat. #M2200S). The ligation product was transformed into competent *E. coli* bacteria and incubated in 500µl of growth media for 45 minutes. The transformed bacteria were then plated on LB agar plates with ampicillin and incubated overnight at 37°C. Single colonies were chosen and amplified in 5mL of LB broth. Recombinant plasmids were purified using Qiagen plasmid mini kits (Valencia, CA). The plasmids were verified for successful ligation by PCR analysis using Crimson Taq Polymerase and agarose gel electrophoresis.

CELL CULTURE

Human glioblastoma cell line LN229 was maintained in DMEM with 10% FBS at 37°C degrees Celsius and 5% CO_2 at 100% humidity. The human foreskin fibroblast line MSU1.1 was maintained in EMEM with 10% FBS at 37 degrees Celsius and 5% CO_2 at 100% humidity. Both cell types were plated and maintained on 10cm culture dishes.

TRANSFORMATION OF BASIGIN-3/MKATE2 RECOMBINANT FUSION VECTOR

The mKate2 expression vector and the basigin-3 mKate2 recombinant fusion vector were transformed into LN229 GBM cell line and the MSU1.1 fibroblast cell line using the Neon transfection system (ThermoFisher, Waltham MA). One confluent 10cm culture plate of each the LN229 and MSU1.1 was treated with trypsin and counted. The cells are diluted to the suggested cell density and the cells are loaded into the Neon system tips. The appropriate settings are programed into the Neon system and the electroporation takes

place (Table 2). The cells are plated on 60mm culture plates after transfection in antibiotic free media. The cells are allowed to grow overnight before media is replaced with media treated with neomycin (G418) as a selection process. The cell media was changed regularly and the cells were passaged when needed. Further selection was made using Bio-rad cell sorter to select for cells expressing the red florescent protein mKate2.

REAL-TIME PCR

Separate 10cm culture plates were prepared for RNA collection for the LN229 untreated cells and the LN229 cells that were transfected with the recombinant fusion protein. Cells were lysed with RLT lysis buffer and the RNA was purified from each sample using the RNAeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized using AMV reverse transcriptase (Promega Cat. #M5101). The real-time PCR (rt-PCR) reaction was performed using SYBR green mastermix. The 19µl of the PCR master mix (SYBR green, primers, dH_2O) was loaded into the 48-well plate with 1µl of cDNA sample. The reaction was perfomed by using an Applied Biosciences StepOnePlus thermocycler and was programed to begin with 1 cycle at 95°C for 10 minutes and then 40 cycles of 15 seconds at 95°C for denaturing, decreased to annealing temperature of 55°C for 1 minute, and increased for elongation at 73°C for 30 seconds.

The threshold cycle (Ct) was calculated using an automatic baseline and were exported to excel. The gene expression was analyzed using a comparative Ct method. This method involves normalization to a housekeeping gene, in this case Gyceraldehyde-3-phosphate dehydrogenase (GAPDH), to calculate a delta Ct. The delta Ct of the transformed cell line with the basigin-3 overexpression is then compared to the delta Ct of the untreated cell line to get the delta delta Ct. Fold change is calculated by 2^(-delta delta Ct). Fold

change indicates if there is any change in gene regulation. A fold change above one indicates an up-regulation and a negative fold change indicates a down-regulation.

RESULTS

TRANSFECTION OF THE EXPRESSION PLASMIDS RESULTED IN THE SUCESSFUL TRANSLATION OF THE MKATE2 PROTEIN.

The plasmids were confirmed for successful insertion of the basigin-3 construct (Figure 1) using a double digestion with mlu1 and sgf1 endonucleases. The basigin-3 insert was ligated into the mKate2 expression vector using PCR primers with endonuclease sites cloned into the end of the basigin-3 sequence. The double digestion confirmed that three of the ligations had basigin-3 construct successfully inserted (Figure 2).

LN229 GBM and MSU1.1 fibroblast cell lines were cultured to confluency and 100,000 were transfected with either the mKate2 or the Basigin-3/mKate2 fusion protein expression vector (Figure 1) using the Neon transfection system. Transfected cells were cultured with the antibiotic neomycin to maintain selection for cells containing the recombinant plasmid for the remainder of the project. Flow cytometry and confocal imaging was used to confirm successful transfection and that there was successful translation of the mKate2 protein in vivo. Flow cytometry was performed using a BioRad (Hercules, CA) Cell sorter. Shown in Figure 3A and D are the untransfected LN229 and MSU1.1, respectively, cell count verses the fluorescence of mKate2 protein (left) and the size of the cells verses the fluorescence (right). These readings were used to gate the baseline fluorescence seen in the cells. In Figure 3B, E show the LN229 and MSU1.1 cells that were transfected with an expression vector with only mKate2. The plots show a distinct shift to the right indicating that there is an increase in red fluorescence, therefore the cells have been successfully transfected and mKate2 is being translated. The cells that

were transfected with the basigin-3 mKate2 construct also show a shift to the right (Figure 3C, G) indicating successful translation of the basigin-3 mKate2 fusion protein.

The confocal images of the transfected cells (figure 4) also confirm that the cells were successfully transfected and the mKate2 was being translated *in vivo*. Consistent with the flow cytometry results the cells transfected with mKate2 only plasmid (center) is expressing more fluorescence than the cells transfected with the basigin-3 mKate2 construct (right). Figure 5 shows separated panels of the LN229 cells transfected with the basigin-3 mKate2 construct. The positive red fluorescence confirms again that the mKate2 protein is being successfully translated within the fusion protein construct. The cells seem to be smaller than usual and unfortunately are not in a good enough shape to see the subcellular localization of the fusion protein.

EXPRESSION OF BASIGIN-3 IN LN229 GLIOBLASTOMA CELL LINE

A PCR reaction with basigin-3 specific primers was run to examine if there is normally basigin-3 expressed in the LN229 GBM line. The middle lane is from the LN229 control sample and the lane on the right is from the LN229 sample transfected with the basigin-3 mKate2 expression vector. The positive bands indicate that there is basigin-3 present in both samples and implies that basigin-3 is normally expressed in the LN229 GBM cell line (figure 6).

mRNA EXPRESSION OF MATRIX MALLOPROTEINASE PROTEINS IN BASIGIN-3 OVEREXPRESSED CELLS

To examine if there are any changes in MMP expression when basigin-3 is overexpressed quantitative real-time PCR was ran on RNA isolated from the LN229 cells transfected with the basigin-3 mKate2 expression vector (Figure 6). These results were averaged from seven replicates of each target gene. Basigin-3 was show at a 1.3 fold change compared to control LN229 cells, a lower expression change than expected. None of the MMP genes we targeted (MMP1, MMP2, and MMP3) showed a decrease in expression in the LN229 cells and there was no significant increase in their expression (Table 3).

DISCUSSION

Construction and overexpression of the basigin-3/mKate2 fusion protein in the LN229 GBM cell line was performed successfully. Analysis of the fusion protein expression using both flow cytometry and confocal microscopy demonstrated that the fusion protein was translated within the cells. Unfortunately, the image quality from the confocal images did not allow for any conclusions to be made on the subcellular localization of basigin-3 in the cells. The results are consistent with the Liao et al., 2011 paper were the authors indicated that basigin-3 is expressed on the cell surface (Liao et al., 2011).

One of the proposed functions of basigin-3 is that it has an inhibitory effect on basigin-2 (EMMPRIN) function and could inhibit the basigin-2 mediated expression of MMPs. In this study, the evidence that suggests the overexpression of basigin-3 does not decrease the expression of MMP1, MMP2, or MMP3 in GBM cell lines. In fact, it appears that MMP2 expression is increased in the cells expressing the basigin-3/mKate2 fusion protein. Additional experiments would need to be performed in order to place any statistical significance to this assertion.

There are several possible explanations for these results. First, it is possible that the presence of the mKate2 red fluorescent fusion protein on the carboxy terminus of the basigin-3 sequence altered the signaling function of the basigin-3 protein. In order to test this, future work using overexpression of un-tagged basigin-3 might rule out this possibility. Secondly, the promoter driving expression of the basigin-3/mKate2 fusion might not drive expression at a high enough level to produce changes in MMP gene expression. In addition, the MMPs that might be responsive to basigin-3 overexpression in GBM cells may not be MMP-1, -2, or -3. The hepatocarcinoma cells used in the Liao et al.

paper are fundamentally different than GBM cells in their origin and cellular location. Thus, it is difficult to know whether this is an inherent limitation of the GBM model being used without further information on the expression of MMPs in GBM cells.

Finally, it is difficult to interpret the results without a more detailed analysis of the interactions between basigin-2 and basigin-3 at the plasma membrane of the cell. The results of this study cannot rule out the possibility that the overexpressed basigin-3/mKate2 construct did not physically interact with basigin-2 (EMMPRIN) at the plasma membrane. Confirmation of such a protein interaction at the plasma membrane requires the use of Forster Resonance Energy Transfer (FRET) methodologies using the basigin-2 and basigin-3 proteins expressed as recombinant fusion proteins. This work was initiated but not completed due to time constraints. However, the initial work of creating and characterizing the basigin-2 and basigin-3 FRET vectors is described in Appendix B.

This study was the first to test the function of basigin-3 overexpression in GBM cell line. Like most scientific work, this study has raised more questions than it has answered. For example, if the overexpressed basigin-3 protein does have an effect upon cell growth, what are these effects? Several possibilities include changes in the ability of the cells to grow under varying oxygen or glucose levels, or changes in gene expression other than MMP-1, -2, or -3. According to the published literature on basigin-3, this isoform is expressed at low levels in normal tissue types. If the observation that basigin-3 expression is elevated in GBM cell lines, this protein would still make an interesting target to study for potential therapeutic approaches.

REFERENCES

Alberts, B. (2008). *Molecular biology of the cell* (5th ed.). New York: Garland Science.

Association, A. B. T. (2010). Brain Tumor Primer: A Comprehensive Introduction to Brain Tumors.

Bachinski, L. L. O., S.; Zhou, X.; Wu, C.; Yip, L.; Shete, S.; Lozano, G.; Amos, C. I.; Strong, L. C.; Krahe, R. (2005). Genetic Mapping of a Third Li-Fraumeni Syndrome Predisposition Locus to Human Chromosome 1q23. *Cancer Research, 65*(2).

Belton, R. J., Jr., Chen, L., Mesquita, F. S., & Nowak, R. A. (2008). Basigin-2 is a cell surface receptor for soluble basigin ligand. *J Biol Chem, 283*(26), 17805-17814. doi:10.1074/jbc.M801876200

Biswas C., Z. Y., DeCastro R., Guo H., Nakamura T., Kataoka H., and Nabeshima K. . (1995). The Human Tumor Cell-derived Collagenase Stimulatory Factor (Renamed EMMPRIN) Is a Member of the Immunoglobulin Superfamily. *Cancer Research*(55), 434-439.

Bleau, A.-M., Huse, J. T., & Holland, E. C. (2014). The ABCG2 resistance network of glioblastoma. *Cell Cycle, 8*(18), 2937-2945. doi:10.4161/cc.8.18.9504

Brems, H. B., E.; de Ravel, T.; Legius, E. . (2009). Mechanisms in the pathogenesis of malignant tumours in neurofibromatosis type 1. *Lancet Oncology, 10*, 508-515.

Brennan, C. W., Verhaak, R. G., McKenna, A., Campos, B., Noushmehr, H., Salama, S. R., . . . Network, T. R. (2013). The somatic genomic landscape of glioblastoma. *Cell, 155*(2), 462-477. doi:10.1016/j.cell.2013.09.034

Coons, S. W. J., P. C., Scheithauer, B. W.; Yates, A. J.; Pearl, D. K. (1996). Improving Diagnostic Accuracy and Interobserver Concordance in the Classification and Grading of Primary Gliomas. *American Cancer Society*.

Duffner F., R. R., Freudenstein D., Weller M., Dietz K., and Wessels J. (2005). Specific intensity imaging for glioblastoma and neural cell cultures with 5-aminolevulinic acid-derived protoporphyrin IX. *Journal of Neuro-Oncology*(71), 107-111.

Dunn, G. P., Rinne, M. L., Wykosky, J., Genovese, G., Quayle, S. N., Dunn, I. F., . . . Hahn, W. C. (2012). Emerging insights into the molecular and cellular basis of glioblastoma. *Genes Dev, 26*(8), 756-784. doi:10.1101/gad.187922.112

Eljamel, M. S., Goodman, C., & Moseley, H. (2008). ALA and Photofrin fluorescence-guided resection and repetitive PDT in glioblastoma multiforme: a single centre Phase III randomised controlled trial. *Lasers Med Sci, 23*(4), 361-367. doi:10.1007/s10103-007-0494-2

Ferner, R. E. (2007). Neurofibromatosis 1. *Eur J Hum Genet, 15*(2), 131-138. doi:10.1038/sj.ejhg.5201676

Gont, A. H., J. E. L.; Lavictoire, S. J.; Parolin, D. A. E.; Daneshmand, M.; Restall, I. J.; Soucie, M.; Nicholas, G.; Woulfe, J.; Kassam, A.; Da Silva, V. F.; Lorimer, I. A. J. . (2013). PTEN loss represses glioblastoma tumor initiating cell differentiation via inactivation of Lgl1. *Oncotarget, 4*(8).

Iacono, K. T., Brown, A. L., Greene, M. I., & Saouaf, S. J. (2007). CD147 immunoglobulin superfamily receptor function and role in pathology. *Exp Mol Pathol, 83*(3), 283-295. doi:10.1016/j.yexmp.2007.08.014

Jin, Y., Bin, Z. Q., Qiang, H., Liang, C., Hua, C., Jun, D., . . . Qing, L. (2009). ABCG2 is related with the grade of glioma and resistance to mitoxantone, a chemotherapeutic drug for glioma. *J Cancer Res Clin Oncol, 135*(10), 1369-1376. doi:10.1007/s00432-009-0578-4

Juratli, T. A., Schackert, G., & Krex, D. (2013). Current status of local therapy in malignant gliomas--a clinical review of three selected approaches. *Pharmacol Ther, 139*(3), 341-358. doi:10.1016/j.pharmthera.2013.05.003

Kirk P., W. M. C., Heddle C., Brown M.H., Barclay A.N., and Halestrap A.P. (2000). CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *The European Molecular Biology Organization Journal, 19*(15), 3896-3904.

Lawrence J. E., B. C., Belton R. J., Rovin R. A., and Winn R. J. (2015). Targeting DNA Repair Mechanisms to Treat Glioblastoma. In C. Chen (Ed.), *DNA Repair*.

Le Floch, R., Chiche, J., Marchiq, I., Naiken, T., Ilc, K., Murray, C. M., . . . Pouyssegur, J. (2011). CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. *Proc Natl Acad Sci U S A, 108*(40), 16663-16668. doi:10.1073/pnas.1106123108

Liao, C. G., Kong, L. M., Song, F., Xing, J. L., Wang, L. X., Sun, Z. J., . . . Chen, Z. N. (2011). Characterization of basigin isoforms and the inhibitory function of basigin-3 in human hepatocellular carcinoma proliferation and invasion. *Mol Cell Biol, 31*(13), 2591-2604. doi:10.1128/MCB.05160-11

Medicine, I. o. (1992). *Advances in Understanding Genetic Changes in Cancer*: National Academy Press.

Pecorino, L. (2012). *Molecular Biology of Cancer: Mechanisms, Targets, and Therapeutics*: Oxford University Press.

Pezzolesi, M. G., Zbuk, K. M., Waite, K. A., & Eng, C. (2007). Comparative genomic and functional analyses reveal a novel cis-acting PTEN regulatory element as a highly conserved functional E-box motif deleted in Cowden syndrome. *Hum Mol Genet, 16*(9), 1058-1071. doi:10.1093/hmg/ddm053

Rivera, A. L., Pelloski, C. E., Gilbert, M. R., Colman, H., De La Cruz, C., Sulman, E. P., . . . Aldape, K. D. (2010). MGMT promoter methylation is predictive of response to radiotherapy and prognostic in the absence of adjuvant alkylating chemotherapy for glioblastoma. *Neuro Oncol, 12*(2), 116-121. doi:10.1093/neuonc/nop020

Roos, W. P., & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol Med, 12*(9), 440-450. doi:10.1016/j.molmed.2006.07.007

Sameshima, T. N., K.; Toole, B. P.; Yokogami, K.; Okada, Y.; Goya, T.; Koono, M.; Wakisaka, S. . (2000). Glimoa Cell extracellar matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activated gelatinase A in co-cultures with brain-derived fibroblasts. *Cancer Letters*(157), 177-184.

Shibahara, I., Sonoda, Y., Saito, R., Kanamori, M., Yamashita, Y., Kumabe, T., . . . Tominaga, T. (2013). The expression status of CD133 is associated with the pattern and timing of primary glioblastoma recurrence. *Neuro Oncol, 15*(9), 1151-1159. doi:10.1093/neuonc/not066

Stupp, R., Hegi, M. E., Mason, W. P., van den Bent, M. J., Taphoorn, M. J., Janzer, R. C., . . . National Cancer Institute of Canada Clinical Trials, G. (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol, 10*(5), 459-466. doi:10.1016/S1470-2045(09)70025-7

T., M. T. M. (2003). Basigin (CD147): a multifunctional transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion. *History and Histopathology Cellular and Molecular Biology, 18*, 981-987.

Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., & Blenis, J. (2003). Tuberous Sclerosis Complex Gene Products, Tuberin and Hamartin, Control mTOR Signaling by Acting as a GTPase-Activating Protein Complex toward Rheb. *Current Biology, 13*(15), 1259-1268. doi:10.1016/s0960-9822(03)00506-2

Tsai, W. C., Chen, Y., Huang, L. C., Lee, H. S., Ma, H. I., Huang, S. M., . . . Hueng, D. Y. (2013). EMMPRIN expression positively correlates with WHO grades of astrocytomas and meningiomas. *J Neurooncol, 114*(3), 281-290. doi:10.1007/s11060-013-1184-5

Wang, K., Yamamoto, H., Chin, J. R., Werb, Z., & Vu, T. H. (2004). Epidermal growth factor receptor-deficient mice have delayed primary endochondral ossification because of defective osteoclast recruitment. *J Biol Chem, 279*(51), 53848-53856. doi:10.1074/jbc.M403114200

Wang, S. I. P., J.; Li, J.; Bruce, J. N.; Cairns, P.; Sidransky, D.; and Parsons, R. (1997). Somatic Mutations of PTEN in Glioblastoma Multiforme. *Cancer Research*(57), 4183-4186.

Weidle, U. H. S., W.; Eggle, D.; Klostermann, S.; and Stockinger, H. (2010). Cancer-related Issues of CD147. *Cancer Genomics and Proteomics*(7), 157-170.

Weinburg, R. (2007). *The Biology of Cancer*: Garland Sciences.

Weller, M., Felsberg, J., Hartmann, C., Berger, H., Steinbach, J. P., Schramm, J., . . . Loeffler, M. (2009). Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. *J Clin Oncol, 27*(34), 5743-5750. doi:10.1200/JCO.2009.23.0805

Yang, M., Yuan, Y., Zhang, H., Yan, M., Wang, S., Feng, F., . . . Wang, L. (2013). Prognostic significance of CD147 in patients with glioblastoma. *J Neurooncol, 115*(1), 19-26. doi:10.1007/s11060-013-1207-2

Zhou, S., Liao, L., Chen, C., Zeng, W., Liu, S., Su, J., . . . Li, J. (2013). CD147 mediates chemoresistance in breast cancer via ABCG2 by affecting its cellular localization and dimerization. *Cancer Lett, 337*(2), 285-292. doi:10.1016/j.canlet.2013.04.025

Zhu, H., Acquaviva, J., Ramachandran, P., Boskovitz, A., Woolfenden, S., Pfannl, R., . . . Charest, A. (2009). Oncogenic EGFR signaling cooperates with loss of tumor suppressor gene functions in gliomagenesis. *Proc Natl Acad Sci U S A, 106*(8), 2712-2716. doi:10.1073/pnas.0813314106

APPENDIX A: FIGURES AND TABLES

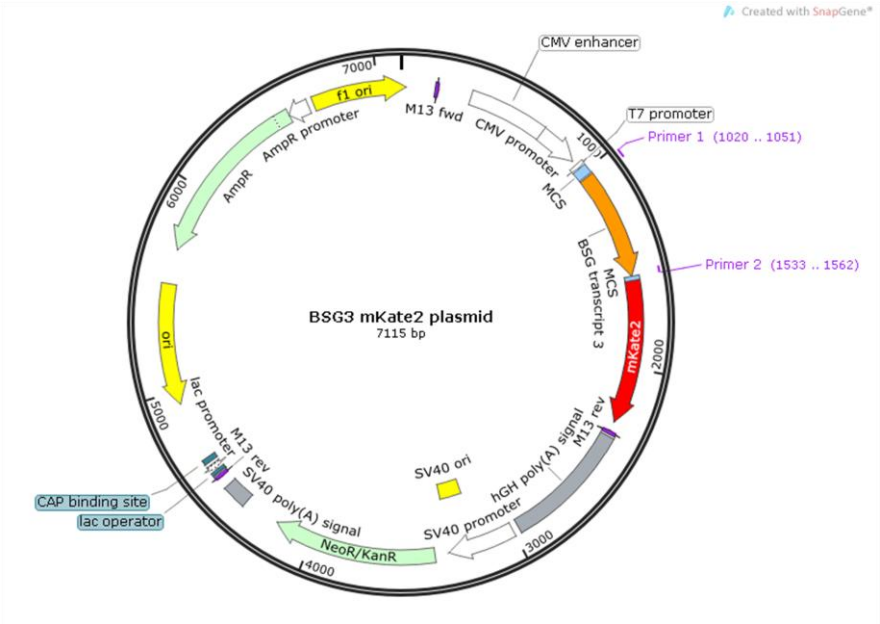


Figure 1. Basigin-3 mKate2 fusion protein plasmid map. Construct was designed with the mKate2 protein to be fused on the C-terminus of the Basigin-3 protein.

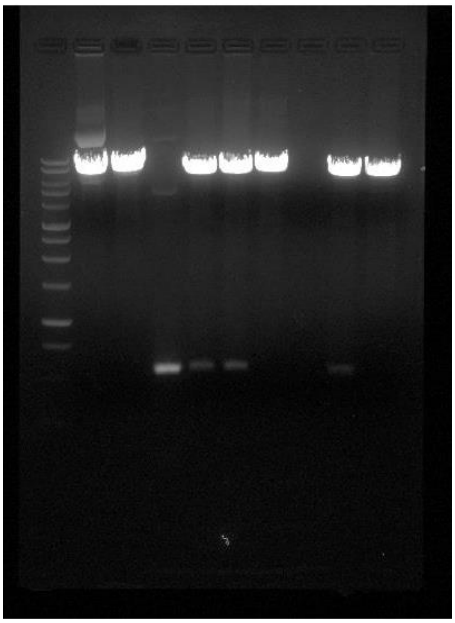


Figure 2. Double digest with mlu1 and sgf1 to confirm successful insertion of Basigin-3 into the plasmid constructs. From left to right: Ladder, plasmid single cut sgf1, Basigin-3 PCR product, plasmid single cut mlu1, double digested ligation 1, ligation 2, ligation 3, ligation 4, ligation 5, ligation 6. Ligations 1,2, and 5 show successful basigin-3 inserts.

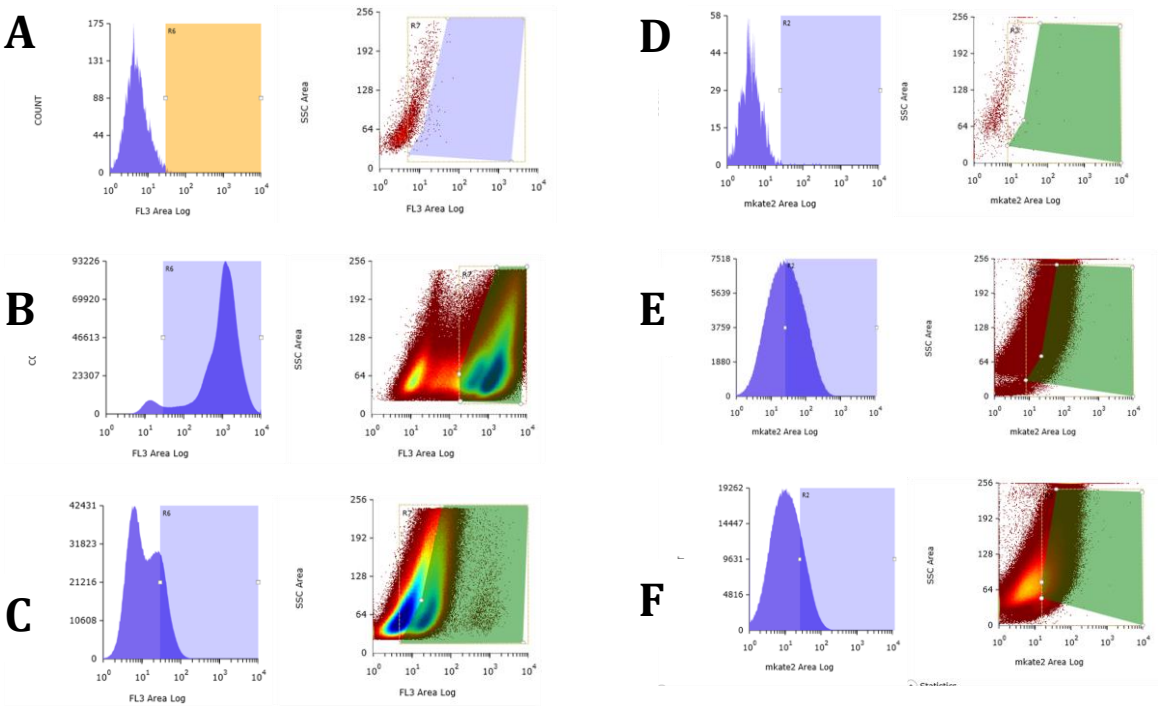


Figure 3. Cell sorter flow cytometry charts confirming successful transfection of both LN229 and MSU1.1 cell types. The graphs on the left of each segment are a cell count vs. fluorescence and the graphs on the right are cell size vs. fluorescence. (A) LN229 GBM control cells. (B) LN229 +mKate2, cells were transfected with a plasmid containing the mKate2 sequence. (C) LN229 + BSG3/mKate2 construct cells that were transfected with the recombinant fusion construct of Baigin-3 mKate2. (D) MSU1.1 fibroblast control cells. (E) MSU1.1 + mKate2, cells were transfected with a plasmid containing the mKate2 sequence. (F) MSU1.1 + BSG3/mKate2 construct cells that were transfected with the recombinant fusion construct of Baigin-3 mKate2.

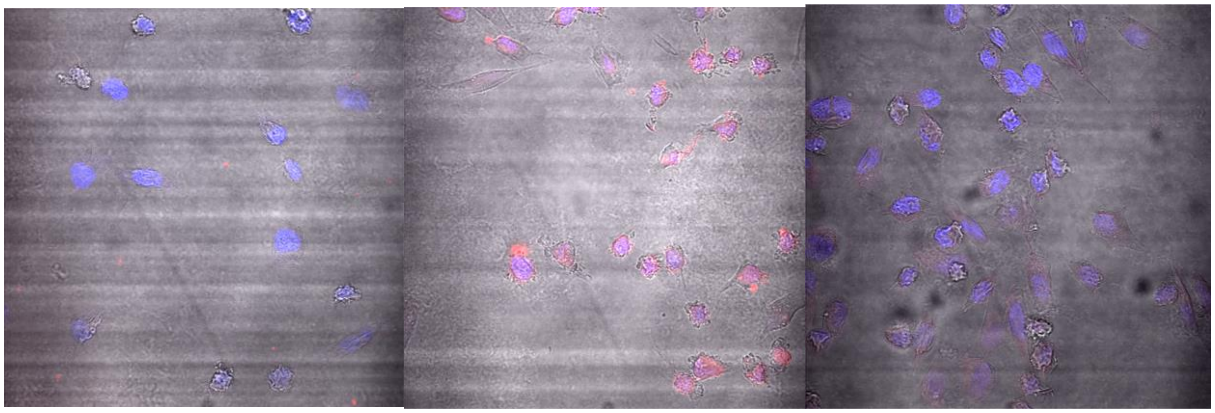


Figure 4. Confocal images of LN229 cell line. From left to right: nontransfected LN229, transfected with with the mKate2 only plasmid, transfected with the Basigin-3 mKate2 fusion protein plasmid.

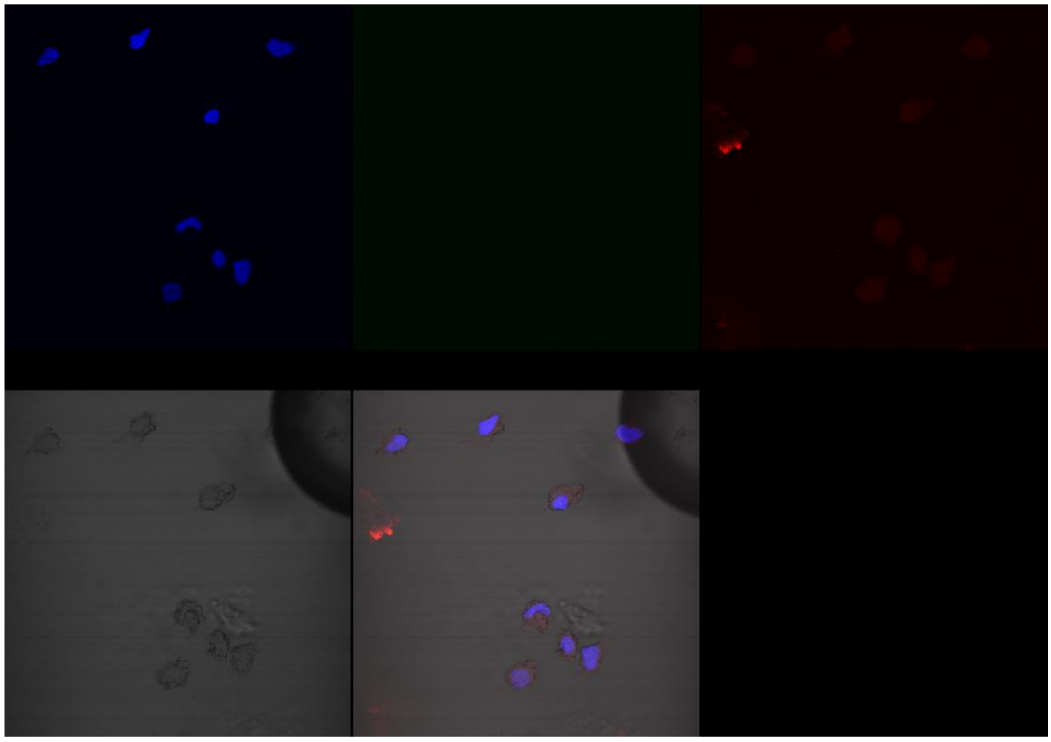


Figure 5. Confocal images of Basigin-3 expression in LN229 cells. Top left to right: DAPI (blue), lipophilic dye (green), mKate expression (red). Bottom left to right: Transmitted light image , all images combined, blank square.

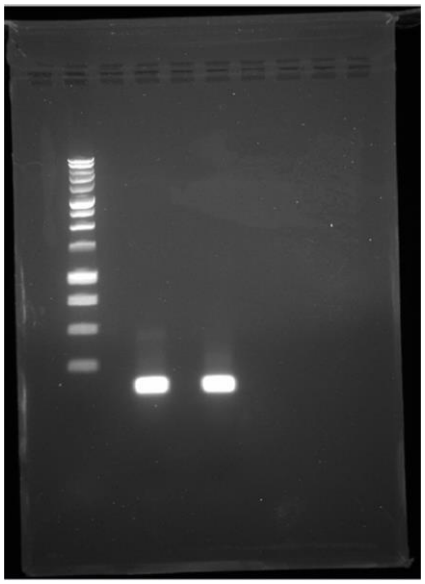


Figure 6. Basigin-3 expression in LN229 GBM cell line. A PCR reaction amplifying basigin-3 in LN229 control cells (left) and the LN229 cells transfected with the basigin-3 mKate2 fusion vector

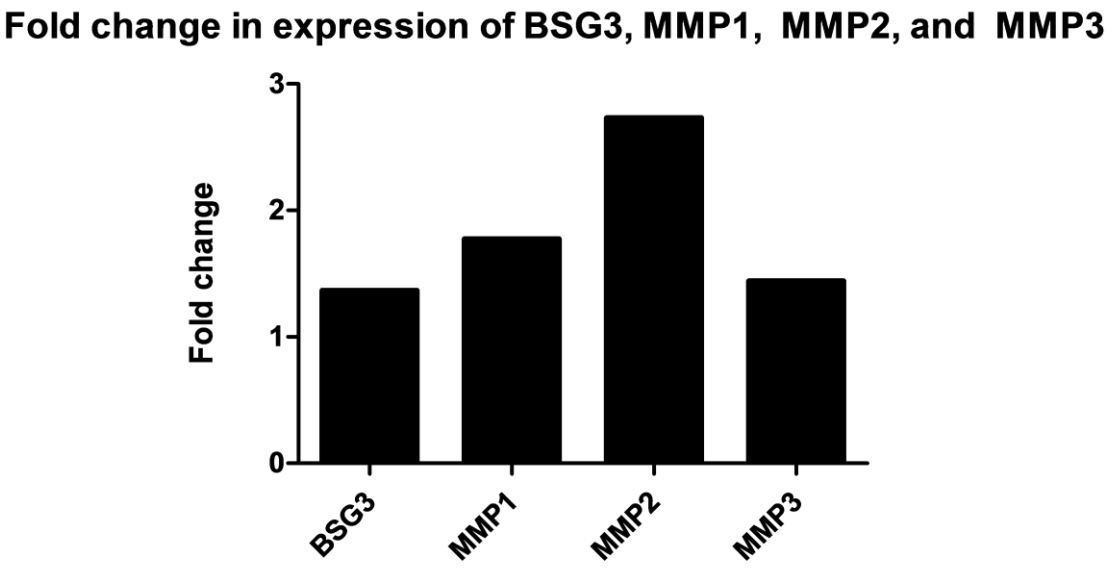


Figure 7. Basigin-3 overexpression does not decrease MMP-1/2/3 expression in LN229 cells. qRT-PCR results support that the increased expression of basigin-3 does not cause an inhibitory effect on the expression of MMP

Table 1: Primer sequences

Primer	Forward primer sequences (5'-3')	Reverse primer sequences (5'-3')	Use of primer
BSG3 mkate2	GCGACGCGTGGAAGAG TTCCTCTGGCGGACGTT	GAGGCGATCGCCATGGGC ACGGCCAACATCCAGCT	Amplification and addition of restriction site on BSG3 for mKate2 vectors
BSG3 FRET	GAATCATGGGCACGGC CAACATCCAGCT	TTGCAGGCGGTCTCCTTG AGGGGACTACCTAGG	Amplification and addition of restriction site on BSG3 for FRET vectors
BSG2 FRET	GAGAATTCAATGGCGG CTGCGCTGTTCGT	TTGCAGGCGGTCTCCTTG AGGGGACTACCTAGG	Amplification and addition of restriction site on BSG2 for FRET vectors
EP-BSG3	CGGCTTAGTCTGCGGTC C	GGGAGGAAGACGCAGGAG TA	PCR verification of BSG3 insert
EP-BSG2	GCGGTTGGAGGTTGTA GGAC	GGGAGGAAGACGCAGGAG TA	PCR verification of BSG2 insert
RT-qBSG3	EP-BSG3 forward primer	CGACCACGGTGAGAAAAG TC	qRT-PCR
MMP1	GGTCTGAGGGTCAAGC AG	AGTTCATGAGCTGCAACA CG	qRT-PCR
MMP2	ACATCAAGGGCATTCA GGAG	GCCTCGTATACCGCATCA AT	qRT-PCR
MMP3	GCAGTTTGCTCAGCCTA TCC	GAGTGTCGGAGTCCAGTT C	qRT-PCR

Table 2: Neon system electroporation parameters

	Voltage (V)	Width (ms)	Pulse #	Cell Density (cells/mL)	Transfection efficiency (%)	Viability (%)
MSU1.1	1650	20	1	$5x10^6$	92	90
LN229	1300	30	1	$5x10^6$	70	70

Table 3: Column statistics of qPCR results.

	Basigin-3	MMP1	MMP2	MMP3
Number of values	7	7	8	7
Minimum	0.1923	0.1701	1.060	0.1851
25% Percentile	0.1923	0.1701	1.060	0.1851
Median	1.139	2.367	1.683	0.3175
75% Percentile	2.301	2.450	5.530	4.382
Maximum	2.301	2.450	6.812	4.382
Mean	1.367	1.775	2.732	1.441
Std. Deviation	0.9558	1.097	2.535	2.010
Std. Error	0.3613	0.4146	0.8963	0.7597
Lower 95% CI of mean	0.4826	0.7604	0.6121	-0.4179
Upper 95% CI of mean	2.251	2.789	4.851	3.300
One sample t test				
Theoretical mean	1.000	1.000	1.000	1.000
Actual mean	1.367	1.775	2.732	1.441
Discrepancy	-0.3665	-0.7748	-1.732	-0.4409
95% CI of discrepancy	-0.5175 to 1.251	-0.2396 to 1.789	-0.3882 to 3.851	-1.418 to 2.300
t, df	t=1.015 df=6	t=1.869 df=6	t=1.932 df=7	t=0.5804 df=6
P value (two tailed)	0.3494	0.1108	0.0947	0.5828
Significant (alpha=0.05)?	No	No	No	No

APPENDIX B: FRET VECTORS

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis 2: Basigin-3 interacts with basigin-2 (EMMPRIN) in GBM cell lines

Aim 2: Examine the protein interactions between basigin-3 and basigin-2 in GBM tumors by the use of FRET analysis.

METHODS

FRET PLASMIDS

The plasmids were purchased from Addgene, mCerulean N1 was a gift from Steven Vogel (Addgene plasmid # 27795), and mVenus N1 was a gift from Steven Vogel (Addgene plasmid # 27793). The mCerulean and mVenus (FRET vectors) were transformed into *E. coli* bacteria and plated with Kanamycin.

Bacteria colonies were allowed to grow overnight before a single colony from each plate was selected and used to inoculate 5mL of LB broth for amplification. After the confirmation of bacteria growth from the small amplifications the 5mL vials were used to inoculate 100mL LB broth for large scale amplification. For all amplifications the LB broth was kept at 37℃ on a shaker.

Plasmids were purified from the bacteria using Qiagen Plasmid mini and maxi kits (Valencia, CA). After purification a nanodrop was used to measure DNA concentration. Restriction enzymes and gel electrophoresis were used to verify plasmid identity. The restriction enzymes EcoR1 and BamH1 were used for the FRET vectors.

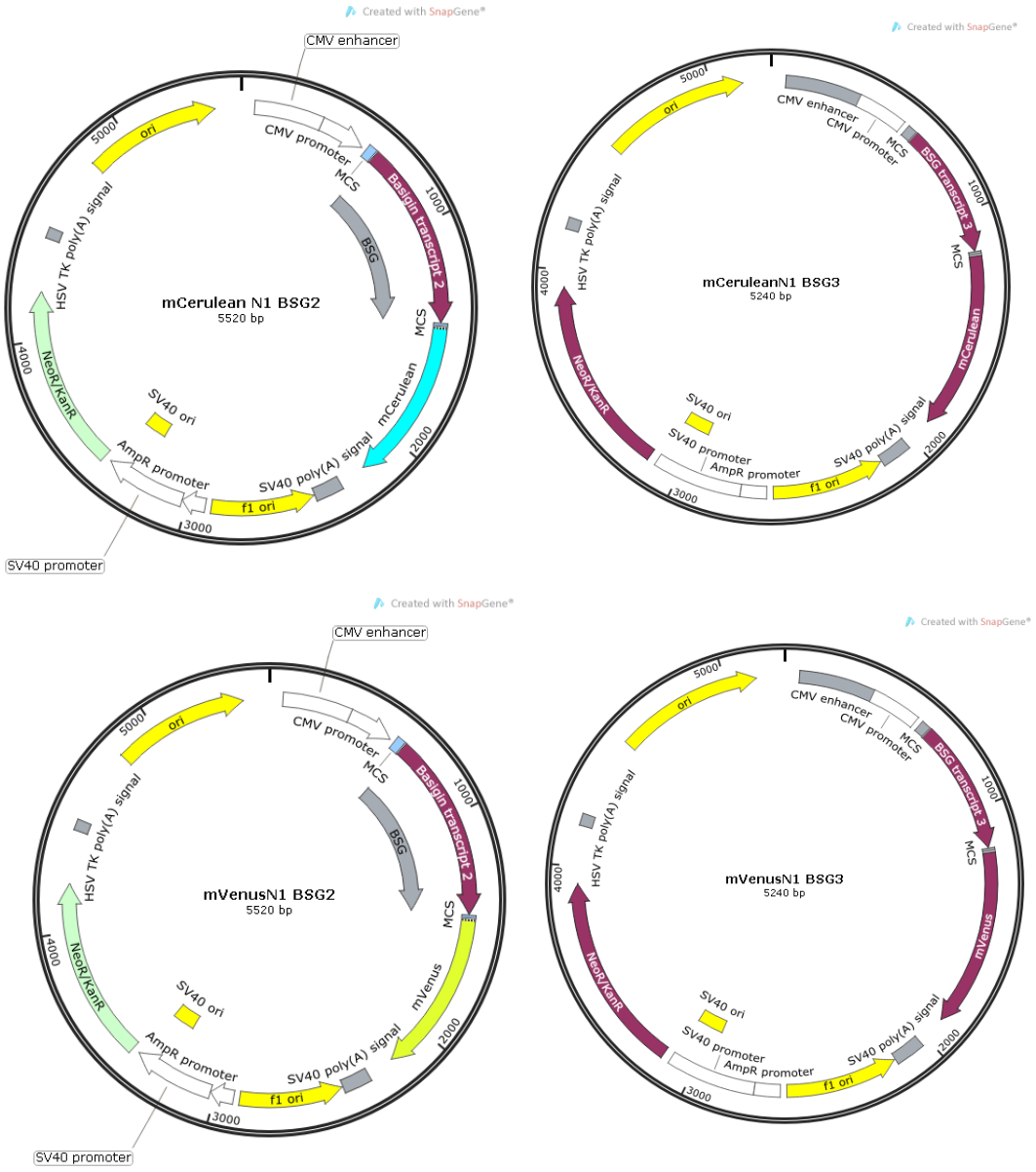
SUBCLONING OF FRET VECTORS

The basigin-3 and basigin-2 insert was amplified using high fidelity Phusion DNA polymerase purchased from ThermoFisher (Waltham MA). Primers were designed to clone in restriction sites on the gene inserts in order to preferentially clone the genes in correct orientation for the FRET expression vectors. Primer information can be found in Table 1. Once the gene inserts were amplified by PCR, the ends were digested with the appropriate restriction enzymes. The gene inserts were verified and purified through gel electrophoresis. The digested PCR product band was cut out of the agrose gel and purified using QIAquick gel extraction kit purchased from Qiagen (Valencia, CA). The mKate2 expression vector was digested using EcoR1 and BamH1 and was also purified through gel electrophoresis.

The basigin-3 gene insert and the basigin-2 gene insert were ligated into each of the FRET expression vectors (mCreulean and mVenus) using New England BioLab's Quick Ligation Kit (Cat. #M2200S). The ligation product was transformed into *E. coli* bacteria and incubated in 500µl of growth media for 45 minutes. The transformed bacteria was then plated on LB agar plates treated with ampicillin and incubated overnight at 37℃. Single colonies were chosen and amplified in 5mL of LB broth.

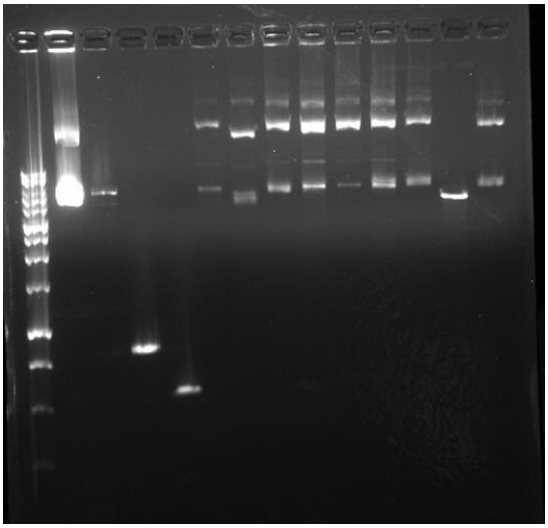
The plasmids of the amplified bacteria were purified using Qiagen plasmid mini kits (Valencia, CA). The plasmids were verified for successful ligation by PCR using Crimson Taq Polymerase and ran through gel electrophoresis. The plasmids with the correct clones are further amplified and purified for transfection. The plasmids where confirmed for

successful insertion of the basigin-3 construct (App. Figure 1) using a double digestion with mlu1 and sgf1 endonucleases. The basigin-3 insert was ligated into the FRET expression vectors using PCR primers with endonuclease sites cloned into the end of the basigin-3 sequence. The double digestion confirmed that three of the ligations had basigin-3 or basigin-2 constructs successfully inserted (App. Figure 2). Ligation 3-2 (mVenus+basigin-2), Ligation 4-2 and 4-5 (mCerulean+basigin-2), Ligation 5-2 (mVenus+Basigin-3), and Ligation 6-5 (mCerulean-Basigin-3) all showed successful inserts from either the double digest experiment or the PCR amplification of the insert (App. Figure 2).

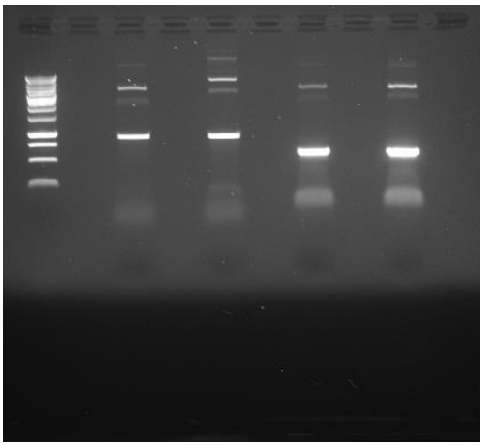


Appendix Figure 1. FRET plasmid maps. A plasmid was created in order for there to be a basigin-3 and an EMMPRIN fusion protein with each of the FRET vectors.

A



B



Appendix Figure 2. Double digest and PCR results. A. Shows the double digest results for the ligation 3 and 4. From the left: Ladder, uncut plasmid, cut plasmid, EMMPRIN PCR product, BSG3 PCR product, Ligation 3 (1-5), ligation 4 (1-4). From this gel you can see that ligation 3-2 and ligation 4-2 run smaller than the other cut plasmids, suggesting that there might be a successful insert of the EMMRIN construct. **B.** Shows the PCR results when Ligation 4-2 and 4-5, ligation 5- 2, and ligation 6-5 were targeted for their respected EMMPRIN or BSG3 inserts. All of the samples came back positive for the insert.