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INVESTIGATING BMP7 EXPRESSION IN GLIOBLASTOMA MULTIFORME

A Thesis

by

YAJAIRA JANETT MACIAS

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

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May 2021

Major Subject: Biology

INVESTIGATING BMP7 EXPRESSION IN GLIOBLASTOMA MULTIFORME

A Thesis
by
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May 2021

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ABSTRACT

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The PI3K/AKT/mTOR pathway regulates important cell processes such as growth, survival, motility, inflammation, proliferation, and apoptosis. In Glioblastoma multiforme (GBM) the PI3K/AKT/mTOR pathway is aberrant as it is almost always active. This results in the deregulation of downstream molecules and ultimately leads to cancer progression and maintenance in GBM tumors. In this study, we used RNA-sequencing to identify differentially expressed genes (DEGs) in U87MG GBM cells treated with NVP-*BEZ235*, a dual inhibitory drug targeting PI3K and mTOR. A total of 7,803 differentially expressed genes were identified via RNA-seq. GEPIA2 online tool was used to assess differential gene expression significance in clinical GBM tumors compared to normal control samples. We also validated the expression of five DEGs, including *BMP7*, in five GBM cell lines using q-RT-PCR. We treated U87MG cells with drugs targeting the PI3K/AKT/mTOR pathway and found no evidence that *BMP7* gene expression is directly regulated by FOXO 1. However, *BMP7* was associated with several differentially expressed genes in U87MG cells in response to NVP-*BEZ235*. Thus, we conclude that *BMP7* expression and regulation should be studied to ultimately find novel genes for targeted drug therapies for GBM treatments.

DEDICATION

This achievement would not have been possible without the unconditional love and support from my family, my husband, and my suri. To my family, thank you for always believing in me. Everything I am, I am because of you. Everything I do, I do for you. To my husband, thank you for supporting me on every step of the way, you and suri lightened even my darkest days.

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I will be forever grateful to Dr. Megan Keniry for accepting me into her laboratory and for mentoring me thoughtout my journey in research. I have always been interested in science but having her as a role model has made an impact in my professional and personal life. Dr. Keniry's guidance and support throughout the past two years, especially throughout the COVID-1 pandemic, has motivated me to work harder and more efficiently. I aspire to be as dedicated and passionate for my future projects as Dr. Keniry is for research.

I also would like to acknowledge my thesis committee members, Dr. Robert Gilkerson, Dr. Matthew Terry, and Bonnie Gunn for their continued support and guidance. I had carried their advice up to the culmination of my research work.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Glioblastoma multiforme (GBM) is arguably the most common and aggressive type of primary brain tumor, it accounts for approximately 48.6% of all malignant central nervous system (CNS) brain tumors and has the highest incidence among all malignant brain tumors: 3.23 cases per 100,000 population (Ostrom et al., 2020). According to the World Health Organization (WHO), GBM falls under the grade IV astrocytoma category given its highly proliferative and infiltrative nature. GBM can develop two ways: *de novo* (also known as primary GBM) or by evolving from a grade I-III tumor to progress to grade IV astrocytoma (also known as secondary GBM). Despite extensive therapeutic research, the median survival rate for GBM patients is 8 months, while the 5-year survival rate only ~ 36%. The prognosis is even worse for GBM patients older than 40 years of age, for whom the 5-year survival rate drops to 21.5% (Ostrom et al., 2020).

GBM is a glial tumor that can appear in the brain, spinal cord, cerebellum, and brain stem, and standard therapy includes tumor resection, radiotherapy/chemotherapy, and stereotactic radiosurgery (SRS). However, current therapy has not been enough to improve patient prognosis and there is no definitive curative treatment for GBM. One of the major challenges for GBM treatment is tumor recurrence, which is highly associated with GBM inter-tumor and intra-tumor heterogeneity. GBM tumor heterogeneity has been targeted with a

molecular therapeutic approach (Inda, Bonavia and Seoane, 2014). Given that ~ 90% of GBM tumors develop *de novo* and considering primary GBM tumors possess lesser tumor homogeneity than secondary GBM tumors (Ohgaki and Kleihues, 2012), it is of pivotal importance to develop new molecular therapies that target tumor heterogeneity to develop more efficacious therapies for GBM.

In GBM tumors, mutations to the phosphoinositide 3-kinase (PI3K)–AKT-mTOR signaling pathway are frequently observed. The main function of the (PI3K)–AKT-mTOR signaling pathway is to regulate cellular processes such as cell maintenance, homeostasis, growth, proliferation. One way the (PI3K)–AKT-mTOR pathway regulates such cell processes is by modulating Forkhead box O transcription factors. These transcription factors regulate the expression of tumor suppressor genes (Zhang, Tang, Hadden and Rishi, 2011). Genetic alterations to diverse members of the (PI3K)–AKT-mTOR signaling pathway result in constitutive activation of the pathway, which in turn results in translocation of FOXO proteins (known tumor suppressor proteins) from the nucleus to the cytoplasm, where these proteins remain in their inactive phosphorylated state and cannot regulate the expression of their target genes. In multiple cancer settings, such rendering of FOXO proteins to the cytoplasm drives tumor progression since tumor suppressor genes and oncogenes cannot be properly regulated. However, in the GBM setting, FOXO protein translocation to the cytoplasm, caused by constitutive activation of the (PI3K)–AKT-mTOR signaling pathway, does not take place. Instead, FOXO proteins remain in the nucleus in their active state. Recently, studies have shown a relationship between poor GBM prognosis and the nuclear localization of FOXO proteins.

In this study, we aim to validate the expression levels of the bone morphogenetic protein – 7 (BMP-7), a member of the transforming growth factor (TGF)-β superfamily that was shown

to be induced in the U87MG cell line after treatment with NVP-*BEZ235*, a dual PI3K/mTOR pathway inhibitor drug. In addition, we hypothesize BMP-7 induction is FOXO dependent.

Given GBM aggressiveness, incidence, and recurrence, which is most frequently observed within 8 months of primary treatment, it is of imperative importance to better understand the molecular interactions that drive cancer progression and maintenance of GBM to further investigate new targeted therapies for the treatment of GBM.

CHAPTER II

LITERATURE REVIEW

Brain tumors - A public health problem

Brain and other type of central nervous system tumors give rise to a variety of symptoms, including frequent headaches, coordination issues, memory loss, seizures, convulsions, speech issues, mood swings, paralysis in one side of the body, vision or/and hearing changes, nausea, vomiting, and disorientation (American Association of Neuroscience [AANS], 2021). There are two categories of brain tumors: primary and secondary. Tumors that arise from brain tissue or tissue from the brain's immediate surroundings are primary brain tumors. In contrast, tumors that develop from elsewhere in the body, lungs or breast for instance, and migrate to the brain are secondary brain tumors. Primary tumor subcategories include glial or non-glial tumors. Further subcategorization of primary brain tumors includes benign (non-cancerous) and malignant (cancerous) tumors (Harvard Health, 2021).

The average annual age-adjusted incidence rate (AAAIR) for brain and other CNS tumors is 23.79 per 100,000 (Ostrom et al., 2020). In 2016, the Central Brain Tumor Registry of the United States (CBTRUS) reported that brain and other CNS tumors were the leading cancer type that causes cancer death among children and adolescents age 0-14. The AAAIR for this age group is 5.8 per 100,000 population. Among adult patients, age 40 and older, brain and other

CNS tumors were the 8th most common cancer type with an AAAIR of 42.85 per 100,000 population. Among primary malignant tumors including Glioblastoma multiforme (GBM), pineoblastoma, medulloblastoma, and ependymoblastoma among others, Glioblastoma multiform is the most commonly occurring tumor accounting for 48% of all malignant brain tumors (Ostrom et al., 2020).

Glioblastoma multiforme (GBM)

Glioblastoma multiforme (GBM), also known as glioblastoma, is a highly invasive glial tumor. GBM tumors occur predominantly in the brain; however, tumors can also appear in the spinal cord, cerebellum, and brain stem. Some characteristics of GBM include rapid growth and spreading into surrounding brain tissue. The World Health Organization (WHO) classifies gliomas in four grades. According to WHO criteria, grade I gliomas fall under the benign tumor category. On the other hand, grade II and III gliomas can invade surrounding brain tissue and progress to grade IV gliomas (Dang, Jin and Su, 2010). GBM is a WHO grade IV astrocytoma tumor that arises from star-shaped glial cells like astrocytes and oligodendrocytes. Although GBM tumors were first thought to arise exclusively from glial cells, new evidence suggests that they can develop from diverse cell types that possess neural stem cell-like properties (Davis, 2016).

Current treatments for GBM include surgery followed by radiotherapy/chemotherapy, and stereotactic radiosurgery (SRS). However, there are multiple challenges for each type of therapy. Surgery (resection of the tumor) in GBM patients is performed not only to obtain a diagnosis, but to remove as much tumor mass as possible to relieve clinical symptoms (Manrique-Guzman, Herrada-Pineda and Revilla-Pacheco, 2021). Tumor resection is followed by radiaotherapy and serves to reduce the need for corticosteroids. Aggressive tumor resection

has been shown to lead to better prognosis, but some challenges include the location of the tumor. Frequently, in GBM patients the tumor is in important areas of the brain that control senses, speech, and motor functions (Davis, 2016). Therefore, the major challenge for surgery as a GBM therapy strategy is GBM tumor recurrence, which, for most patients, occurs only after approximately 8 months of primary treatment (Mallick, Benson, Hakim and Rath, 2016). Radiotherapy (RT) treatment for GBM, after tumor resection, has been shown to improve overall survival and involves a dosage of 60 Gy in 1.8 to 2.0 Gy fractions (Batash et al., 2017). However, tumor recurrence also represents a challenge for radiotherapy since it usually resurges at the tumor's primary site, a site that most likely has received maximal therapy already. Tumor location was the original challenge, the new challenge is migration of malignant cells into adjacent brain tissue since it impedes the use of local therapy (Ryu et al., 2014). Concurrent radiation treatment with chemotherapy is current standard therapy for GBM. Chemotherapy involves temozolomide (TMZ), alkylating agent, oral or intravenous administration (Batash et al., 2017). Chemotherapy by itself does not prevent tumor recurrence, evidence suggests this is caused by the characteristic GBM tumor heterogeneity, which indicates certain tumor subpopulations are resistant to TMZ treatment (Auffinger et al., 2015). In a study performed in 2005, it was demonstrated that concurrent chemoradiotherapy was more effective than just post-operative radiotherapy (Stupp et al., 2009). Stereotactic radiosurgery is a non-invasive form of therapy that involves the use of an external beam to treat tumors by delivering localized irradiation. Unfortunately, this type of treatment was not shown to increase survival (Ryu et al., 2014).

As previously described, these therapy approaches have their own challenges, with tumor recurrence being the common denominator among them. This has led researchers to investigate

other types of therapies that aim to prevent tumor recurrence. By investigating the genomic profile of GBM tumors researchers aim to target GBM subpopulations that are resistant to different therapies.

GBM tumors are subcategorized using both the tumor's genotype and phenotype. These tumors develop in the brain in one of two ways; *de novo* or by evolving from lower-grade astrocytomas or oligodendrogliomas; WHO grade I-III tumors. GBM tumors arising *de novo* with no identifiable precursor genetic or phenotypic lesion are also known as primary glioblastomas, while GBM tumors developing from lower-grade tumors are known as secondary glioblastomas (Louis et al., 2021). For GBM tumors, there are two genotypic categories; IDH-wildtype and IDH-mutant glioblastomas. GBM tumors lacking the *IDH* genetic mutation in genes *IDH1* (*Isocitrate Dehydrogenase 1*) and *IDH2* (*Isocitrate Dehydrogenase 2*) are classified as IDH-wildtype and GBM tumors with IDH mutation fall into the IDH-mutant classification. As of 2016, approximately 90% of GBM cases are IDH-wildtype while the remaining 10% of cases are IDH-mutant. IDH-wildtype tumors are predominantly found in the supratentorial area of the brain in patients age 55 and older, necrosis in this type of tumor is extensive. In contrast, IDH-mutant tumors arise more commonly in the frontal lobe of the brain in children and adolescent patients age 0-19 (Louis et al., 2021).

Evidence shows that IDH genetic lesions occur at high frequencies in grade II gliomas such as astrocytomas and olingodendromas, 68% and 69% *IDH1* mutation frequency. Additionally, a high frequency of IDH lesions is observed in grade IV secondary GBM with an 88% *IDH1* mutation frequency. This suggests that in the onset of secondary GBM tumors, *IDH1* mutations play an important role in oncogenesis (Dang, Jin and Su, 2010). Genetic lessons in *IDH* genes are not the only significant genetic disturbances in GBM. Genetic alterations vary in

primary vs. secondary GBM tumors (Crespo et al., 2015). In primary GBM tumors three main genetic alterations are predominant. (1) high *EGFR* (Epidermal Growth Factor Receptor) gene mutation frequency in chromosome 7p; amplification and/or mutation of *EGFR* is found in 36-60% of primary GBM tumors. Variant 3 (*EGFRvIII*) is the most common type of *EGFR* mutant; it leads to a constitutively active protein, and its overexpression contributes to cancer proliferation and survival in GBM. (2) *CDKN2A*-p16 homozygous deletion in chromosome 9p; loss of homozygosity resulting in deletion of tumor-suppressor *CDKN2A* (Cyclin Dependent Kinase Inhibitor 2A) gene is found in 31-78% of primary GBM tumors. (3) Loss of heterozygosity (LOH) of chromosome 10; LOH results in deletion of chromosomal region 10q23-24, which encodes for the expression of tumor-suppressor *PTEN* (Phosphatase and Tensin homologue) gene. Interestingly, although LOH of chromosome 10 is found in 70% of primary GBM tumors, mutated *PTEN* gene has been found in only 25%. Additionally, LOH in chromosome 10 is associated with poor prognosis while *PTEN* mutation is only slightly associated with increased survival, suggesting the presence of other tumor-suppressor genes encoded by other deleted regions in chromosome 10 such as deleted region 10q14-15 and 10q25pter. Other frequent genetic alterations in primary GBM tumors include the amplification of *MDM2* (Mouse Double Minute 2 Homolog) oncogene, the mutation/homozygous deletion of *NF1 (*Neurofibromin 1), and mutations on *PI3KR1* (regulatory subunit 1 of phosphatidylinositol 3-kinase) gene, found in 15%, 18% and 10%, respectively, of all primary GBM tumors (Crespo et al., 2015).

As previously discussed, high *IDH1* mutation frequency is one of the major genetic lesions associated with secondary GBM tumors. However, there are other major genetic alterations associated with secondary GBM tumors. (1) *TP53* (Tumor Protein p53) gene

frequency mutations at chromosome 17p; mutations are found in approximately 65% of secondary GBM tumors. *TP53* mutations are commonly found at the onset of secondary GBM, suggesting it participates in malignant transformation from primary to secondary GBM. (2) Partial LOH of chromosome 10q, and complete LOH of chromosomes 13q, 19q, and 22q. LOH of Chromosome 13q frequently results in loss of the *RB* locus and inactivation of tumor suppressor genes (Crespo et al., 2015).

In GBM, epigenetic alterations including abnormal DNA methylation, chromatin remodeling, histone modifications, and miRNAs expression have been found to contribute to tumor formation (Crespo et al., 2015). O6-methyl guanine methyltransferase (MGMT) involvement in DNA repair is important to repair genetic alterations to prevent mutations. However, in GBM hypermethylation on the promoter region of MGMT is found in approximately 68% of GBM samples. Given MGMT's pivotal involvement in DNA repair, its silencing through hypermethylation results in higher mutation frequencies in GBM, which is associated with poor prognosis (Burgess, Jenkins and Zhang, 2008). Chromatin remodeling through post-translational modifications such as methylation, acetylation, phosphorylation ubiquitination, sumoylation and poly(ADP)-ribosylation of histones is associated with carcinogenesis. Such alterations to the chromatin structure can impact the recruitment of proteins involved in DNA repair.

In recent years, evidence has shown that multiple microRNAs play a significant role in gene regulation. Although abnormal expression of microRNAs, including *miR-128*, *miR-181a*, *miR-181b*, *miR-181c*, and *miR-221*, and *miR-21* has been found in GBM, little is known about how these abnormalities contribute to GBM carcinogenesis. Interestingly, studies show that overexpression of *miR-21* is associated with decreased apoptosis in GBM, and that inhibition of

miR-21 induces apoptosis, suggesting that it might act as an oncogene by regulating of genes involved in invasion and migration (Gabriely et al., 2008 & Burgess, Jenkins and Zhang, 2008).

Resources like genomic profiling and information from the Cancer Genome Atlas project have revealed three main signaling pathways that are commonly mutated in GBM tumors. The tumor suppressor protein p53 pathway is commonly lost. The receptor tyrosine kinase [RTK]/Ras/phosphoinositide 3-kinase [PI3K]–AKT signaling pathway is almost always activated. The retinoblastoma gene (*RB*) is mutated to an inactive form (Furnari et al., 2007). Novel therapies targeting some of these genetic alterations have been proposed as treatment for GBM.

Receptor tyrosine kinase (RTK)/Ras/phosphoinositide 3-kinase (PI3K)–AKT-mTOR signaling pathway.

The phosphoinositide 3-kinase (PI3K)–AKT signaling pathway plays a significant role in intracellular processes such as glucose metabolism, cell growth and survival, motility, inflammation, and proliferation. The phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases localized in the plasma membrane. These PI3Ks are categorized into three classes; class I, class II, and class III (Martini et al., 2014). Class I PI3Ks are implicated in cancer, while little is known about class II and III PI3Ks. Class I PI3Ks enzymes are heterodimeric and consist of two subunits: a catalytic subunit, p110, and a regulatory subunit (Yang et al., 2019). In mammals, four isoforms for the catalytic subunit p110 have been discovered: α, β, γ, and δ. In addition, multiple regulatory subunits include the p85, p101, and p84/p87. The regulatory subunit p85 is most commonly associated with catalytic subunits p110α, p1100β and p110δ. Regulatory subunits p101, and p84/p87 are associated with p110γ (Martini et al., 2014).

Mammalian PI3Ks can phosphorylate phosphatidylinositols (PtdIns) to transduce signals received from activated Ras sarcoma (RAS) signaling pathway or from different activated membrane receptors such the tyrosine kinase receptors (RTKs), and the G-coupled receptors (GPCRs). Canonically, upon receptor activation PI3K is recruited to the cell membrane, where interaction between the intracellular section of the activated receptor and the PI3K regulatory subunit takes place. Such interaction allows for the catalytic subunit to associate with the lipid membrane to phosphorylate phosphatidylinositol-4,5-biphosphate (PIP_2) to phosphatidylinositol-3-4,5-triphosphate (PIP_3). PIP_3 then acts as a second messenger molecule that represents an anchor site for proteins that possess a pleckstrin homology domain. One of these proteins includes the AGC kinase phosphoinositide-3-kinase-protein kinase B/AKT, which is recruited upon PI3K activation and translocated to the inner membrane. AKT activation is dependent on phosphorylation by the Ser/Thr 3-phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates AKT at Thr308. AKT phosphorylation and further activation is enough to activate downstream mammalian target of rapamycin complex 1 (mTORC1). Activation of both AKT and mTORC1 results in increased protein, nucleotide, and lipid synthesis, which in in turn promotes cell proliferation by inducing cell survival and blocking apoptosis. For complete (PI3K)/AKT signaling pathway activation the activity of another mTOR complex is required. mTORC2 phosphorylates AKT at Ser473. Complete AKT activation following mTORC2 phosphorylation results in substrate-specific phosphorylation events downstream AKT. mTORC2-mediated AKT phosphorylation can impact over 20 AKT substrates. Among these substrates the pro-apoptotic transcription factors Forkhead Box subfamily O members FOXO -1, -3 and -4 are found. Upon complete AKT activation these transcription factors are phosphorylated and further sequestrated to the cytoplasm, where they remain in their

phosphorylated inactive form. Over the years studies have shown that some mTORC2 cellular functions include chemotherapy resistance, increased cell survival, invasiveness, and maintenance of glioblastoma stem-like cells (Crespo et al., 2015; Mecca, Giambanco, Donato and Arcuri, 2018; Martini et al., 2014; Majewska and Szeliga, 2016) Under normal conditions, tumor suppressor PTEN negatively regulates the (PI3K)/AKT signaling pathway by dephosphorylating substrate PIP_3 to produce PIP_2 (Martini et al., 2014).

In human cancers, including Glioblastoma multiforme, the phosphoinositide 3-kinase (PI3K)–AKT signaling pathway is significantly deregulated (Hoxhaj & Manning, 2019). Constitutive activation of this signaling pathway results in unrestrained cell proliferation and inactivation of pro-apoptotic pathways. Thus, activating PI3K promotes glioma formation. Diverse factors including mutations to upstream (PI3K) regulators such as RTKs and KRAS, somatic mutations in the *PIK3CA* gene, which codes for the PI3K catalytic subunit p110, and loss-of-function of tumor suppressor *PTEN* gene contribute to upregulation of the (PI3K)–AKT signaling pathway in tumor cells (Bagrodia et al., 2012). Another common (PI3K)/AKT/mTOR mutation associated with GBM includes oncogenic *PIK3R1* gene, which encodes for the p85α regulatory subunit. As previously discussed, loss of *PTEN*, evidenced by low *PTEN* expression levels in multiple brain tumor types, leads to PIP_3 accumulation. Importantly, elevated PIP_3 levels simulate growth factor signaling. Canonically, such growth factor stimulation is triggered by RTK ligands, insulin, or other growth factors (Keniry and Parsons, 2008).

Considering that current therapies for GBM have limitations, it is imperative to better understand how targeting of different (PI3K)/AKT/mTOR signaling pathway members might affect tumor progression and recurrence to serve as a potential therapeutic target for GBM. Multiple studies have shown that inhibition of (PI3K)/AKT/mTOR signaling pathway members

combined with single or dual drugs is associated with decreased TMZ resistance (Haas et al., 2018), increased radiosensitivity (Millet, Granotier, Etienne and Boussin, 2013), and strong antitumor activity (de la Peña et al., 2006). Although much work has been done to better understand how the (PI3K)/AKT/mTOR pathway promotes GBM progression, and how pathway inhibition might serve as a therapeutic approach, there is still poor understanding about the intricate molecular mechanisms that driving these two outcomes.

The phosphoinositide 3-kinase (PI3K)–AKT-mTOR signaling pathway - FOXO transcription factors.

Forkhead box O transcription factors are part of the Forkhead transcription factors family. There are four highly conserved FoxO transcription factors: *FOXO1*, *FOXO3*, *FOXO4* and *FOXO6* (Huang, and Tindall, 2007). FoxO transcription factors play a significant role in the modulation of cellular differentiation, survival, growth, cell cycle, metabolism, stress and tumor suppression pathways (Zhang, Tang, Hadden and Rishi, 2011). The expression of these transcription factors can be found in diverse mammalian tissues. For example, *FOXO1* expression is found in adipose tissue, *FOXO4* is expressed in skeletal muscle, *FOXO3* is expressed in brain, hearth, kidney, and spleen, and *FOXO6* is expressed in the developing and adult brain (Zhang, Tang, Hadden and Rishi, 2011). The transcriptional function of FOXO proteins is regulated by diverse signaling networks such as the insulin signaling pathway. Additionally, FoxO factors are regulated by post translational modifications such as phosphorylation, acetylation, and ubiquitination (Keniry et al., 2013).

FOXO proteins are regulated by the phosphatidylinositol 3-kinase (PI3K)/AKT axis, where phosphorylation of three highly conserved residues occurs. Interestingly, while *FOXO1*, *FOXO3* and *FOXO4* phosphorylation modifications occur on three residues, *FOXO6* phosphorylation

modification occurs on two residues only. As a result of these post-transcriptional modifications FOXOs inactivation occurs (Coomans de Brachène and Demoulin, 2015). These FOXO transcription factors canonically localize to the cytoplasm in response to growth and survival factors such as insulin and insulin-like growth factor 1 (IGF-1). In contrast, FOXO transcription factors localize in the nucleus in response to stress events, regardless of the presence or absence of growth factors (Huang, and Tindall, 2007). Even though AKT is the arguably the best characterized kinase that regulates FOXO transcription factors, other kinases act as negative regulators as well. For example, casein kinase 1 (CK1), dual-specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A), extracellular signal-regulated kinase (ERK), and serum and glucocorticoid-regulated kinase (SGK) (Coomans de Brachène and Demoulin, 2015).

As previously discussed, evidence demonstrates that cell proliferation and survival during carcinogenesis is regulated by the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway (Greer and Brunet, 2005), and previous works has documented dysregulation of the (PI3K)/AKT signaling pathway in cancer. There is an abundance of evidence that suggests that FOXO transcription factors, which are regulated by the (PI3K)/AKT signaling pathway, have a tumor suppressor role on diverse types of cancer (Fu and Tindall, 2008).

When the (PI3K)/AKT signaling pathway is active, FoxO transcription factors are phosphorylated by AKT and translocated to the cytoplasm, where they remain in their inactive state. FoxO factor inactivation then results in cell survival and proliferation, since FoxOs regulate cell cycle and pro-apoptotic target genes such as *TRAIL* (TNF superfamily member 10), *BIM* (Bcl-2-like protein 11), *FAS* (TNF Receptor Superfamily, Member 6), *BCL-6* (B-Cell

Lymphoma 6 Protein) and *CDKN1B* (Cyclin Dependent Kinase Inhibitor 1B) (Huang and Tindall, 2007).

Interestingly, the role of FoxO transcription factors is multifaceted in different cancer settings. In pancreatic cancer, successful dual inhibition of the (PI3K)/AKT and MAPK/ERK signaling pathways result in FOXOs translocation to the nucleus and further FoxO transcription factors activity. Such activity then leads to expression of genes involved in cell cycle arrest and apoptosis. In this cancer setting FOXO proteins ultimately act as tumor growth mediators by inhibiting angiogenesis, which involves cell migration and is crucial for tumor growth (Roy, Srivastava and Shankar, 2010). Mice *in vivo* studies where simultaneous conditional deletion of *FOXO1, FOXO3* and *FOXO4* genes induce tumor formation further supports the tumor suppressor role of FOXO proteins (Paik et al., 2007). In other cancer settings, including acute myeloid leukemia (AML), soft tissue sarcoma and breast cancer, *FOXO1* depletion/downregulation is highly associated with poor prognosis (Farhan et al., 2020). In addition, it has been demonstrated that low expression levels of *FOXO1, FOXO3* or *FOXO4* is correlated with cancer angiogenesis and progression in hepatocellular cancer (Yamaguchi et al., 2013), renal carcinoma (Wu et al., 2013), and prostate cancer (Modur, Nagarajan, Evers and Milbrandt, 2002). This evidence further alludes to the tumor suppressor role of FOXO proteins.

Contrastingly, the tumor promoting role of FOXO proteins has been demonstrated by a variety of studies. In a gastric cancer setting the overexpression of *FOXO3a* promoted cell migration and invasion, while *FOXO3a* suppression by knockdown caused the opposite effect and resulted in reduced migration and invasion (Yu et al., 2016). Other studies suggesting the tumor promoting role of FOXO proteins show a correlation between upregulation of FOXO1 phosphorylation and better gastric cancer prognosis (Kim et al., 2007). In several other cancer

settings, including AML, colorectal and breast cancer, pancreatic ductal adenocarcinoma, and Glioblastoma multiforme, FOXO3 upregulation has been shown to be correlated with poor prognosis by promoting metastasis and invasion (Farhan et al., 2020).

There are instances, depending on the cancer setting, including Glioblastoma multiforme, where FoxO transcription factors are found to be localized in the nucleus even when the PI3K/AKT signaling pathway is constitutively active.

Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily. For epithelial, immune, and neuronal cells, members of the TGF-β signaling pathway play an important role in the regulation of cell differentiation, apoptosis, and proliferation (Seoane et al., 2004). In addition, TGF-β signaling pathway members regulate different developmental processes in multiple types of tissues, as they are involved in embryonic development, inflammatory response, organ formation, and immune function (TGF-beta signaling pathway - Cusabio, 2021).

Several studies have shown the involvement of approximately 20 BMPs in different cellular processes such as differentiation, proliferation, lineage commitment, maintenance, survival, apoptosis, and patterning/morphogenesis (Thawani et al., 2010). Multiple studies have provided insight into the BMP signaling pathway and have demonstrated that BMPs are able to activate not only the canonical BMP-SMAD signaling pathway but other non-canonical signaling pathways as well. Some BMP-activated non-canonical signaling pathways include the (PI3K)/AKT, MAPK/ERK, Janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor kappa B (NF-κB) signaling pathways. BMPs are particularly

known to play a key role in bone and cartilage formation (Thawani et al., 2010). However, recent studies suggest their participation in malignancies. BMPs role in malignancies is attributed to their ability to either induce cell differentiation in pluripotent progenitor cells or promote cell proliferation, depending on the target cell type and malignancy setting.

Interestingly, BMPs have almost paradoxical effects on cancer. BMPs such as BMP-4, -6, -7, -8, -9, -10, -11, -13, and -15 have been identified as biomarkers for prognosis of different types of cancer. For instance, in a breast cancer context, high BMP- 4 mRNA and protein expression levels are highly associated with tumor migration and progression (Guo, Huang and Gong, 2011). However, in breast cancer cell lines with low BMP-6 expression levels and where E-cadherin loss of function is known to be correlated to tumor progression, BMP-6 was identified to rescue E-cadherin expression (Du et al., 2009), suggesting the tumor promoter/suppressor role of diverse BMPs is context dependent. In ovarian cancer, BMP-9 acts as tumor stimulator, it promotes tumor progression and maintenance by promoting cell proliferation. However, BMP-9 in a prostate cancer context, acts as tumor suppressor by inducing apoptosis. Evidence suggests that the paradoxical effects of BMPs are explained by the involvement of BMPs on different molecular events that take place in tumorigenesis or/and metastasis. Such events include epithelial-to-mesenchymal transition, cancer stem-like cell maintenance, and angiogenesis (Zhang et al., 2016). In addition to its potential role as tumor promoter/suppressor proteins, BMPs have been shown to participate in insulin secretion and the development of endocrine pancreas (Chattopadhyay, Singh, Gupta and Surolia, 2017). Moreover, BMPs have been correlated with neural induction, neural tube patterning, regionalization of the brain, eye development regulation, and with neuronal cell processes such as lineage

determination and cell apoptosis in the peripheral nervous system. It has also been proposed that BMPs play a role in somite and limb patterning, and tooth and gut development (Hogan, 2021).

Bone morphogenetic protein – 7 (BMP-7)

Bone morphogenetic protein – 7 (BMP-7), also known as osteogenic protein (OP)-1 (OP-1), is one of the > 20 identified proteins from the bone morphogenetic protein family. Consequently, BMP-7 is a member of the transforming growth factor (TGF)-β superfamily. BMP-7, as many of the other BMPs, is best known for its involvement in the regulation of bone and cartilage formation and homeostasis. However, BMP-7, along with BMP-4, is known to play a key role in neural patterning. Evidence indicate that BMP-7 is involved in the formation of both the central and peripheral nervous system during embryogenesis (Hogan, 2021). Additionally, is has been implicated that BMP participates in the regulation of diverse neural cellular processes. Furthermore, it is eluded that BMP-7 also regulates neuronal maturation (Tate et al., 2012). In addition, multiple studies suggest BMP-7 plays a role in cardiac protection and in the conversion of human pancreatic exocrine cells into insulin producing endocrine cells (Hogan, 2021). Given the involvement of BMPs in neuronal maturation and its involvement in nervous system regulation, including the regulation of neural stem cells and their progenitor cells, BMP7 has been proposed as potential therapeutic for treatment of glioblastoma stem-like cancer cells (GSCs) to induce differentiation (Tate et al., 2012). GSCs secrete BMPs to promote differentiation of tumor cells. However, GSCs secrete inhibitors such as GREMLIN1 to prevent their own differentiation to maintain their own self renewal (Yan et al., 2014).

CHAPTER III

METHODOLOGY

Cell lines, cultures, and drug treatment

Human Glioblastoma (GBM) cells lines U87MG, LN-18, U-118MG, DBTRG and LN229 were acquired from the American Type Culture Collection (Manassas, VA, USA). Cell lines were grown in 10 cm dishes and maintained in standard cell culture growth conditions; in Minimal Essential Media Eagle (MEM) at 37 °C and supplemented with 5% CO_2, 5% anti-fungal anti-bacterial treatment and 10% fetal bovine serum (FBS). U87MG, LN-18, U-118MG, DBTRG and LN229 control group cells were treated with DMSO vehicle. Treatment groups for each cell line were exposed to a clinical 50nM dosage of NVP-*BEZ235* for five days; NVP-*BEZ235* is a dual PI3K/mTOR pathway inhibitor drug acquired from Sigma-Aldrich in St. Louis, MO for three days. U87MG cell culture plates were also exposed to other six different treatments: Rapamycin; an mTOR inhibitor, MK2206; and Akt inhibitor, BKM120; a class IA PI3K inhibitor, AS1842856; a *FoxO1* inhibitor, and dual NVP-*BEZ235*/AS1842856 treatment. Rapamycin and BKM120 were acquired from Fisher Scientific, USA. MK2206 was sourced from Apexbio. AS1842856 was acquired from Calbiochem, Danvers, MA. All drugs were dissolved in DMSO. Control group cells for all treatment conditions were treated with DMSO vehicle. Cells for each treatment group were exposed to 10 nM of rapamycin, 1 µM of MK2206, 1 µM of BKM120, and 200 nM of AS1842856.

RNA Isolation

Total RNA from both the control and drug-treated groups were extracted with the MiniPrep RNAeasy isolation kit on column DNase digestion for 15 minutes, acquired from Qiagen in Hilden, Germany. A NanoDrop spectrophotometer, purchased from ThermoFisher, USA, was used to test the quality of the total RNA isolate samples. RNA isolate samples were kept frozen at a -80 degrees Celsius.

RNA-Sequencing

RNA isolate samples of three biological replicates of each condition, DMSO or NVP-*BEZ235*-treated (1μM for 4 days) in U87MG cells, were outsourced to Novogene Corporation Inc. in the University of California at Davis for RNA-seq analysis. At Novogene, the RNA-seq analysis included sequence assembly, analysis of differentially expressed genes (DEGs), and mapping and alignment to the human genome. The RNA-seq analysis data served to identify differentially expressed genes (DEGs) associated with both DMSO and NVP-*BEZ235*-treated samples.

cDNA Preparation

RNA isolate samples were heated to 65 degrees Celsius to remove secondary structures and transformed into cDNA via thermocycling utilizing Superscript Reverse Transcriptase II, acquired from Invitrogen, Carlsbad, CA. cDNA samples were then stored in freezing conditions at -80 degrees Celsius until further utilization.

Quantitative Real-Time Polymerase Chain reaction (Q-RT-PCR)

Forward and reverse primer sequences were designed using the NCBI genetic data information and primer3 (v 0.4.0) (https://bioinfo.ut.ee/primer3-0.4.0/). Primer sequences were then outsourced to Sigma-Aldrich in Saint Louis, MO, and primers were ordered. Diluted cDNA samples consisted of cDNA, distilled water, diluted primers, and Power SYBR Green Master Mix (from Applied Biosystems in Foster City, CA). PCR analysis, to analyze for gene amplification and expression, was performed using the Applied Biosystems StepOne Real-Time PCR Systems, acquired from Foster City, CA. Gene expression levels were normalized to *ACTIN B*, a housekeeper gene, and calculated using $2^{-\Delta\Delta CT}$ method. Exported PCR data was analyzed using MS Excel to calculate for average, standard error, and t-test.

Validation of DEGs and Bioinformatics analysis

Preliminary studies focused on nineteen of the most differentially expressed genes (DEGs) obtained from the comparative RNA-seq analysis of the DMSO and NVP-*BEZ235*-treated U87MG cells. The Gene Expression Profiling Interactive Analysis (GEPIA2; http://gepia2.cancer-pku.cn/#index) is an online application that utilizes data from The Cancer Genome Atlas (TCGA) database and contains 163 and 207 GBM and normal brain samples, respectively. GEPIA2 was used to filter significantly expressed genes in GBM clinical samples as compared to normal brain samples by ANOVA. To assess gene significance, box plots comparing the expression levels of each gene in GBM and normal brain samples were generated by GEPIA2. In addition, the box plots served to identify the regulation patterns of significantly expressed DEGs, which served to designate genes as either up regulated and down regulated

genes in GBM clinical samples. A p-value < 0.05 is designated as statistically significant. The GEPIA2 screening server to funnel the number of genes selected for further examination.

Furthermore, cross examination of the significantly expressed DEGs was performed via Pearson's correlation analysis using GEPIA2. Survival curves for each gene were generated using GEPIA2 survival analysis tool to determine the relationship between *BMP7*, *SOX2*, or *OCT4* and the prognosis in GBM patients. The survival analysis tool plotted the overall survival (OS) curves of *BMP7*, *SOX2* and *OCT4*. The OS analysis gives insight into the association between high or low expression of a specific gene and the prognosis in GBM patients. Statistically significant difference is designated using a p-value < 0.05.

CHAPTER IV

RESULTS

RNA-Sequencing: Differentially Expressed Gene Analysis

Data from the bioinformatic analysis performed at Novogene provided RNA sequence information, genome mapping and alignment, and a comparative analysis of most differentially expressed genes. Data from the RNA-sequencing analysis, which analyzed mRNA extracted from DMSO (control)- and NVP-*BEZ235*- treated U87MG samples, served to identify the differentially expressed genes (DEGs) between control and NVP-*BEZ235* treated samples. The RNA-sequence analysis identified approximately 7,803 DEGs between the control and NVP-*BEZ235* sample sets. From the list of DEGs a set of genes possibly involved in epithelial to mesenchymal transition (EMT) were selected for further examination. Table 1 shows the list of genes and their respective -log2fold change and p-values. All genes selected for further examination had a p-value greater than 0.05.

Expression patterns of NVP-BEZ235-induced Genes in GBM Clinical samples

We chose to investigate developmental genes that were induced or repressed by NVP-BEZ235 treatment in U87MG cells for further analysis to examine their expression in GBM tumors (from clinical samples of human GBM patients) as compared to normal controls using GEPIA2, an online application that determines gene expression based on information from The Cancer Genome Atlas (TCGA) database. Table 2 depicts the differential expression for indicated

genes in GBM clinical samples. Of the 19 selected genes, 11 genes were not differentially expressed in GBM tumors compared to the control samples by ANOVA, while the remaining 8 genes were found significantly differentially expressed in GBM tumors (compared to normal controls). From the 8 significantly expressed genes, the *BMP7*, *VIM*, *VGF*, *GDAP1L1* and *SNAI2* genes were selected for further analysis. GEPIA2 was used to analyze the expression patterns of these 5 genes. Three genes, *BMP7*, *VGF*, and *SNAI2*, were found to be significantly up regulated in GBM tumors as compared to normal control samples. The other two genes, *VIM* and *GDAP1L1*, were found to be down regulated in GBM tumors as compared to normal control samples. These results are shown in Table 3, along with a brief description of each gene and their respective -log2fold change and p-values from the RNAseq analysis. Figure 1 shows the relative gene expression of selected genes *BMP7*, *VIM*, *VGF*, *GDAP1L1* and *SNAI2* in DMSO and NVP-*BEZ235* treated samples. The data used to generate the graphs was harvested from the RNA-seq analysis of DEGs. From the comparative analysis of DEGs, the data suggest that all five genes are differentially expressed in the U87MG cell line by NVP- *BEZ235* treatment.

Quantitative Real-Time Polymerase Chain reaction (Q-RT-PCR)

Further examination of these genes included q-RT-PCR screening to validate the gene expression of all five DEGs in the U87MG and to examine their gene expression in 4 additional GBM cell lines: LN229, LN-18, U-118MG, DBTRG. Forward and reverse primers for selected genes were generated by Primer3 (v0.4.0). Primer sequences are shown in Table 3. q-RT-PCR screening showed that *BMP7* expression was significantly induced in the U87MG, U-118MG, LN-18, and LN-229, but not in the DBTRG cell line, in response to NVP-*BEZ235* treatment. *BMP7* relative gene expression is shown in Figure 2 A-E and Figure 3. This information provides confidence in the RNA-seq analysis. *BMP7* expression was shown to be induced by

NVP-*BE235* treatment in both the RNA-seq analysis and q-RT-PCR screening. *VIM*, *SNAI2* and *GDAP1L1* expression was shown to be induced by NVP-BE235 treatment in the LN-18, U-118MG, and DBTRG cell lines. *SNAI2* and *GDAP1L1* expression was also enriched in the U87MG, but not in the LN-229, cell line. *VIM* expression was not found to be significantly induced in the U87MG cell line. *VGF* expression was found to be significantly induced by NVP-*BEZ235* treatment in the U87MG, LN-229, and U-118MG, but not in the LN-18 and DBTRG, cell lines (Figure 2A-E). Overall, validation of induced gene expression by NVP-*BEZ235* treatment was achieved by q-RT-PCR screening of the following genes: *BMP7*, *VGF*, *GDAP1L1*, and *SNAI2*.

Pearson Correlations were performed between *BMP7* and *VIM*, *VGF*, *GDAP1L1* and *SNAI2* using GEPIA2 (Table 5) to examine whether they were co-expressed. Results indicated *VIM*, *VGF*, *GDAP1L1* and *SNAI2* were co-expressed with *BMP7*. Moving forward, *BMP7* was selected for further examination.

To further delineate how *BMP7* was regulated by the PI3K Pathway, we treated with drugs to PI3K or downstream effectors molecules and assessed its gene expression. BKM120 drug inhibited class IA PI3Ks, rapamycin drug targeted and inhibited mTOR, MK2206 inhibited Akt, and the AS1842856 (FOXO1-i) drug inhibited FoxO1. *BMP7* expression was analyzed by q-RT-PCR screening. The results indicate *BMP7* gene expression was not induced by BKM120 treatment (Figure 4A) and only slightly, but not significantly, induced by rapamycin, MK2206 and AS1842856 (FOXO 1-i) (Figure 4 B-D). *BMP7* gene expression was also analyzed under dual NVP/AS1842856 (FOXO 1-i) treatment by q-RT-PCR screening. Results indicate *BMP7* relative gene expression is slightly, but not significantly, down-regulated by dual NVP/AS1842856 (FOXO 1-i) treatment (Figure 5).

GEPIA2 Bioinformatics analysis of FOXO 1-regulated genes *SOX2* and *OCT4*

To examine *BMP7* regulation in the PI3K Pathway we investigated the expression patterns of two FOXO 1-regulated genes, *SOX2* and *OCT4* as well as their gene correlation to *BMP7* using the GEPIA2 correlation analysis tool. The box plots generated by GEPIA2 to delineate expression patterns of all three genes demonstrated that *BMP7* and *SOX2* were significantly up regulated in GBM tumor samples (compared to normal samples), while *OCT4* expression was not differentially expressed in GBM tumor samples (compared to normal samples) (Figure 6 A). A correlation analysis was performed to investigate whether the expression of the two FOXO 1-regulated genes, *SOX2* and *OCT4*, was correlated to *BMP7* expression in GBM clinical samples. Results showed that *SOX2* expression was significantly correlated to *BMP7* expression. However, *OCT4* expression was not significantly correlated with *BMP7* in GBM clinical samples. Pearson correlation analysis plots are shown in Figure 6 B. Moreover, to examinate the association between *BMP7*, *SOX2*, and *OCT4*, and the prognosis in GBM patients, an overall survival analysis was performed using GEPIA2. None of the three genes were shown to be significantly associated with better or worse outcome in GBM patients (p-value > 0.05) (Figure 6 C).

CHAPTER V

DISCUSSION

Glioblastoma multiforme (GBM) is the most common and aggressive brain cancer. The incidence rate is increasing with an aging population in the United States with 16,830 GBM deaths in 2018 compared to 40,920 breast cancer deaths in the same year. The prognosis for treated and non-treated GBM patients is poor. Both median survival- and 5-year survival- rates are particularly low compared to more common cancers such as breast cancer (Ladomersky et al. 2019). Even when patients undergo aggressive tumor resection, chemotherapy treatment, or combined tumor resection followed by chemotherapy or/and radiotherapy, the median overall survival rate in the United States is 16-20 months (Ladomersky et al. 2019). The 5-year survival rate drops to even lower for patients that are younger than nine years of age or older than 40 years of age (Ostrom et al., 2020). Despite the enormous efforts made to develop new and more efficacious therapies to treat GBM, no treatment cures GBM. In recent years, as new technologies have been developed in the genomics field, information about the genetic profile of GBM has expanded rapidly. Because of this, researchers can identify genetic aberrations that serve as a point of departure for the investigation of intricate molecular mechanisms that drive cancer initiation, progression, maintenance, and recurrence in GBM patients. It has been reported by the The Cancer Genome Atlas (TCGA) research network, using genome screening and RNA sequencing, that interpatient intra- and inter- tumoral heterogeneity exist in GBM patients. Thus, enhanced understanding about the involvement of different genes involved in mechanisms

driving GBM is pivotal for the development of more targeted therapies that can serve to improve the commonly unfavorable prognosis in GBM patients. Genome screening has identified the PI3K/AKT-mTOR pathway as a significantly deregulated pathway as it is almost always active. It has been reported that, in a cancer setting, the PI3K/AKT/mTOR signaling axis drives cancer proliferation by promoting abnormal cell growth and survival, regulating metabolism, and promoting angiogenesis (Hoxhaj & Manning, 2019). Thus, focusing on molecular components from the PI3K/AKT/mTOR axis confers potential targets for new drug development. This research focuses on investigating and evaluating the involvement and clinical relevance of several genes shown to be differentially expressed in U87MG samples in response to NVP-*BEZ235* treatment compared to control samples. NVP-BEZ235 is a drug that targets the PI3K/AKT/mTOR signaling pathway. Investigating these genes can potentially lead to the discovery of novel targets for GBM treatment.

Here, initially we used Illumina RNA-seq technology to sequence the RNA from three biological replicates under DMSO or NVP-*BEZ235* treatment in multiple GBM cells lines to obtain a comprehensive understanding of the molecular interactions associated with PI3K/AKT/mTOR axis inhibition. The RNA-seq analysis served to identify 7,803 differentially expressed genes between the NVP-*BEZ235* and DMSO-treated U87MG cells. From the 7,803 DEGs nineteen genes (Table 1) were selected for our initial bioinformatics analysis. Using GEPIA2, clinical significance assessment of each gene was evaluated. The box plots generated by GEPIA2 served to discard 11 genes: *BARX1*, *CDH3*, *IL36B*, *KCP*, *EPPIN*, *GLI1*, *KRT81*, *PADI4*, *RASSF9*, *SLITRK6*, and *WISP2*. These genes were not significantly differentially expressed in GBM tumor samples (compared to normal brain samples) (Table 2). Moving forward, we used of the box plots generated by GEPIA2 to determine the expression patterns of

5 genes: *BMP7*, *VIM*, *VGF*, *GDAP1L1* and *SNAI2* (Table 3). Given their diverse functions in different cancer settings, we focused our investigation in these five genes. *VIM*, also known as vimentin, codes for a type III intermediate filament protein and has been designated as a biomarker for epithelial-mesenchymal transition (EMT) (Kang et. al., 2019). Studies have revelated that *VIM* silencing mitigates migration and invasion of GBM cells. Using GEPIA2 we demonstrate that *VIM* is overexpressed in GBM tumor samples compared to normal control samples (Table 3). Interestingly, as shown by the RNA-seq analysis (Figure 1 B) and the q-RT-PCR screening (Figure 2 A-D), *VIM* expression was induced by NVP-*BEZ235* treatment in multiple GBM cell lines. This information can be used for further investigation of *VIM* to understand its regulation by the PI3K/AKT pathway. *VGF*, nerve growth factor inducible, exact involvement in a GBM cancer setting is still unknown. However, it is thought to be involved in the survival of glioblastoma stem-like cells (GSCs) and the growth of differentiated glioblastoma cells (Wang et al., 2018). In this study, we show that *VGF* expression is differentially downregulated in GBM tumor samples (compared to normal control samples) and is inconsistently upregulated in multiple GBM cell lines in response to NVP-*BEZ235* treatment. *GDAP1L1*, also known as ganglioside induced differentiation associated protein 1 like 1, is a gene involved in neural development and has been associated with cell differentiation in mouse (Genecards.org., 2021). However, its involvement in GBM and regulation by the PI3K/AKT/mTOR pathway is still unknown. In this study show that *GADP1L1* is downregulated in GBM tumors (compared to normal control samples) (Table 3), and consistently significantly induced in GBM cell lines in response to NVP-*BEZ235* treatment (Figures 1 E and Figure 2 A-E). *SNAI2*, an oncogenic transcriptional repressor, overexpression has been correlated to GBM cell survival (Yang et. al, 2010). In this study, through GEPIA2 comparative analysis between

GBM tumor samples and normal control samples, we confirm that *SNAI2* is upregulated in GBM clinical samples (Table 3). We also validated *SNAI2* induction in GBM cell lines in response to NVP-*BEZ235* treatment through q-RT-PCR screening (Figure 2 A-E). *BMP7* is a bone morphogenetic protein -coding gene that codes for BMP-7. Previous studies elucidate BMP7 impact in glioblastoma stem-like cells (GSCs), cells that possess stem like properties that promote GBM progression and recurrence. Here, we assessed *BMP7* expression pattern in GBM tumor samples compared to normal control samples and demonstrated that *BMP7* is significantly overexpressed in GBM tumor samples. We validated the RNA-seq results (Figure 1 A) for *BMP7* through q-RT-PCR screening (Figure 2 A-E and Figure 3). *BMP7* gene expression was shown consistently induced in GBM cell lines in response to NVP-*BEZ235* treatment.

We decided to limit out research to investigating the expression and regulation of *BMP7* due to the knowledge gap there is for how *BMP7* is regulated by the PI3K pathway and what its involvement is in GBM. We tried different drug treatments targeting the PI3K pathway and downstream effector molecules. Our results showed that *BMP7* relative gene expression was statistically unaffected by drugs inhibiting mTOR, Akt, class IA PI3K, or FoxO1. However, *BMP7* relative gene expression was slightly downregulated by the FOXO 1-inhibitor drug treatment in U87MG cells.

We then investigated the impact dual NVP-*BEZ235*/AS1842856 (FOXO 1-i) treatment on BMP7 gene expression in U87MG cells to delineate if *BMP7* expression was FOXO 1-dependent. Our results indicates that *BMP7* expression was not significantly impacted by dual NVP-*BEZ235*/AS1842856 (FOXO 1-i) treatment, suggesting *BMP7* expression was not dependent on FOXO 1. We aimed to corroborate this information and made Pearsons

Correlations between *BMP7* and two FOXO 1 -regulated genes, *SOX2* and *OCT4*. *BMP7* expression was significantly correlated to *SOX2* expression in GBM tumors compared to normal control samples. However, it was not significantly correlated to *OCT4*. This information further supports that *BMP7* expression, although it is slightly impacted by AS1842586 treatment, is not FOXO 1 dependent. The overall survival analysis plots showed that *BMP7* expression is not statistically correlated to GBM outcomes.

Ultimately, here we demonstrate that *BMP7* is statistically overexpressed in GBM tumor samples compared to normal control samples. We did not have evidence that *BMP7* expression was FOXO 1 – dependent. However, BMP7 was one of the strongest induced genes in our RNA-seq. experiment in which U87MG cells were treated with NVP-*BEZ235*. Evidence supports this regulation. *BMP7* expression in GBM was not associated with either favorable or unfavorable prognosis in GBM. However, *BMP7* expression was significantly correlated with genes involved in cancer progression like *SOX2*. *BMP7* should be further analyzed to delineate its regulation within the PI3K/AKT/mTOR pathway.

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APPENDIX

TABLES AND FIGURES

Table 1: List of several differentially expressed genes from RNA-Seq Analysis.

Gene	Ensembl ID	Log_2 fold Change NVP/DMSO	*P*-value
BARX1	ENSG00000131668	4.6855	< 0.05
BMP7	ENSG00000101144	4.5266	< 0.05
CDH3	ENSG00000062038	1.3865	< 0.05
DMKN	ENSG00000161249	5.1894	< 0.05
GDAP1L1	ENSG00000124194	3.8591	< 0.05
IL36B	ENSG00000136696	3.8077	< 0.05
KCP	ENSG00000135253	3.8617	< 0.05
MDK	ENSG00000110492	3.6504	< 0.05
DDIT4	ENSG00000168209	-3.2498	< 0.05
EPPIN	ENSG00000101448	-3.313	< 0.05
GLI1	ENSG00000111087	-3.0558	< 0.05
KRT81	ENSG00000205426	-3.2934	< 0.05
PADI4	ENSG00000159339	-3.2768	< 0.05
RASSF9	ENSG00000198774	-3.2011	< 0.05
SLITRK6	ENSG00000184564	-3.2524	< 0.05
SNAI2	ENSG00000019549	1.1286	< 0.06
VGF	ENSG00000128564	1.5593	< 0.05
VIM	ENSG00000026025	0.97428	< 0.05
WISP2	ENSG00000064205	4.4254	< 0.05

Table 2: Significance assessment of differentially expressed genes in GBM tumors (compared to control samples). Gene expression significance in GMB tumors was assessed using GEPIA2 online application based on information from The Cancer Genome Atlas (TCGA) database.

Gene	Expression in GBM tumors
BARX1	not significant
CDH3	not significant
IL36B	not significant
KCP	not significant
EPPIN	not significant
GLI1	not significant
KRT81	not significant
PADI4	not significant
RASSF9	not significant
SLITRK6	not significant
WISP2	not significant
BMP7	significant
DMKN	significant
GDAP1L1	significant
MDK	significant
DDIT4	significant
SNAI2	significant
VGF	significant
VIM	significant

Table 3: List of NVP-*BEZ235*-regulated genes from the RNA-seq. that were significantly differentially expressed in GBM tumors. Log_2 fold changes and p-values were harvested from the RNA-seq analysis, while expression significance assessment of genes in GBM tumors was carried out using GEPIA2 online application. *BMP7*, *VIM*, and *SNAI2* were found to be upregulated in GBM tumors compared to control samples. *VGF* and *GDAP1L1* were downregulated in GBM tumors compared to control samples.

Gene	Description	Log_2 fold Change NVP/DMSO	*P*-value	Expression in GBM tumors
BMP7	bone morphogenetic protein	4.5266	6.12E-33	Upregulated
VIM	vimentin	0.97428	2.08E-11	Upregulated
VGF	VGF nerve growth factor inducible	1.5593	5.57E-38	Downregulated
GDAP1L1	ganglioside induced differentiation associated protein 1 like 1	3.8591	1.38E-38	Downregulated
SNAI2	snail family transcriptional repressor 2	1.1286	1.34E-32	Upregulated

Table 4: List of forward and reverse primers of significantly expressed genes in GBM.

Gene	Forward (5' - 3')	Reverse (5' - 3')	Amplicon size (bp)
BMP7	TCAACCTCGTGGAACATGAC	GTTCCCGGATGTAGTCCTTG	192.3
VIM	CGAAAACACCCTGCAATCTT	CTGGATTTCCTCTTCGTGGA	188.3
VGF	CTTCCTGGGGAGAGTTCCAG	GACACTCCTTCCCCGAACTT	187.7
GDAP1L1	CTCCATGATCCCCAAGTACG	TCTTGGCCATGAGCTTCTTT	186.6
SNAI2	TTCGGACCCACACATTACCT	GCAGTGAGGGCAAGAAAAAG	182.9

Table 5: Table 5: Pearson correlation analysis of differentially expressed genes in GBM tumors (clinical human samples). Correlation analysis was performed using GEPIA2 online application.

Gene	Gene	Correlation coefficient (R)	*P* value
BMP7	*VIM*	0.21	< 0.05
BMP7	*VGF*	0.23	< 0.05
BMP7	*GDAP1L1*	0.16	< 0.05
BMP7	*SNAI2*	-0.23	< 0.05

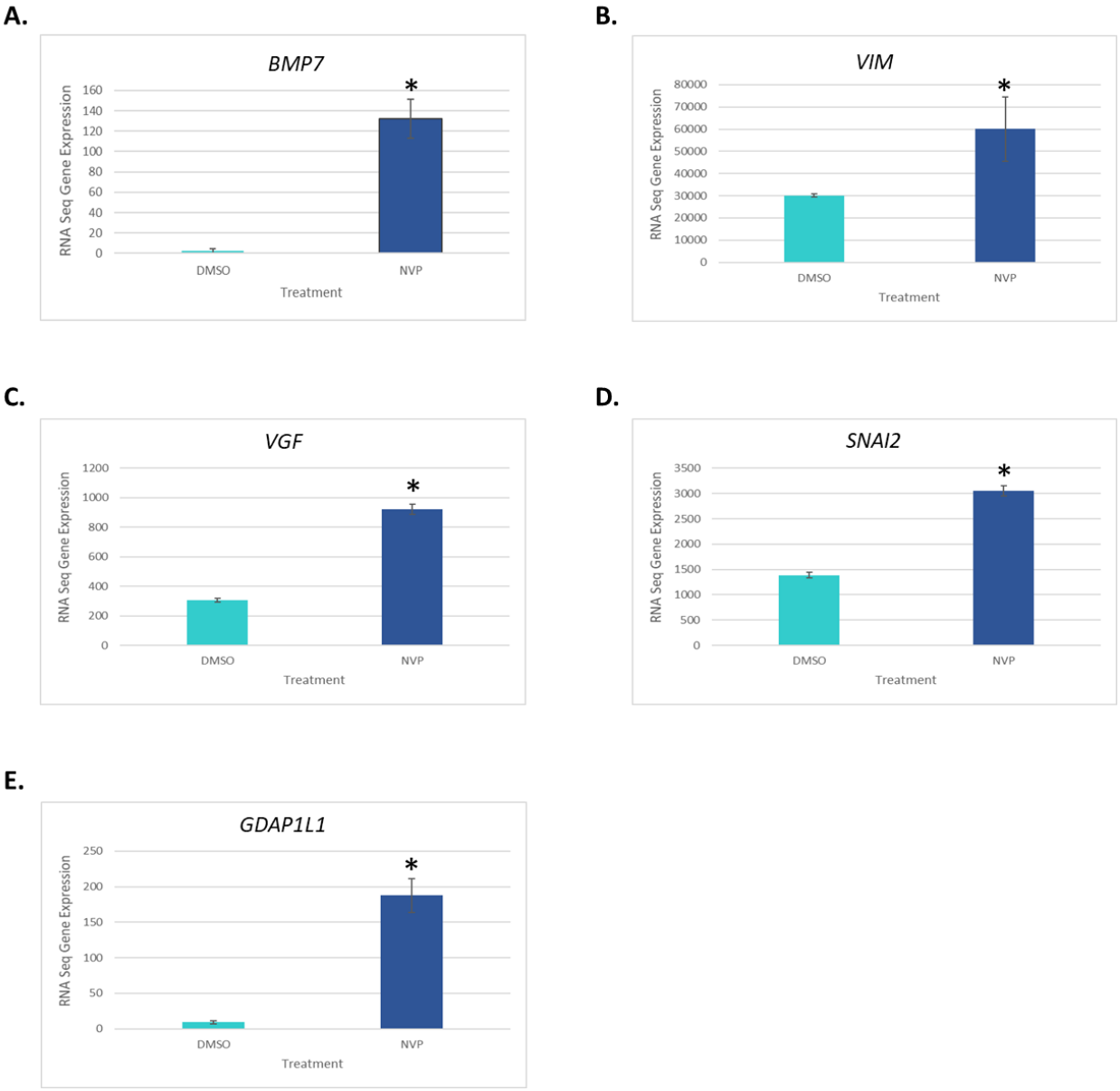

Figure 1: Significantly differentially expressed genes in GMB tumors. (A-E) Differentially expressed genes between DMSO and NVP-*BEZ235* treated samples: *BMP7*, *VIM*, *VGF*, *SNAI2*, and *GDAP1L1*. The relative gene expression of differentially expressed genes was acquired from the RNA-seq analysis. Gene expression significance in GMB tumors was assessed using GEPIA2 online application based on information from The Cancer Genome Atlas (TCGA) database.

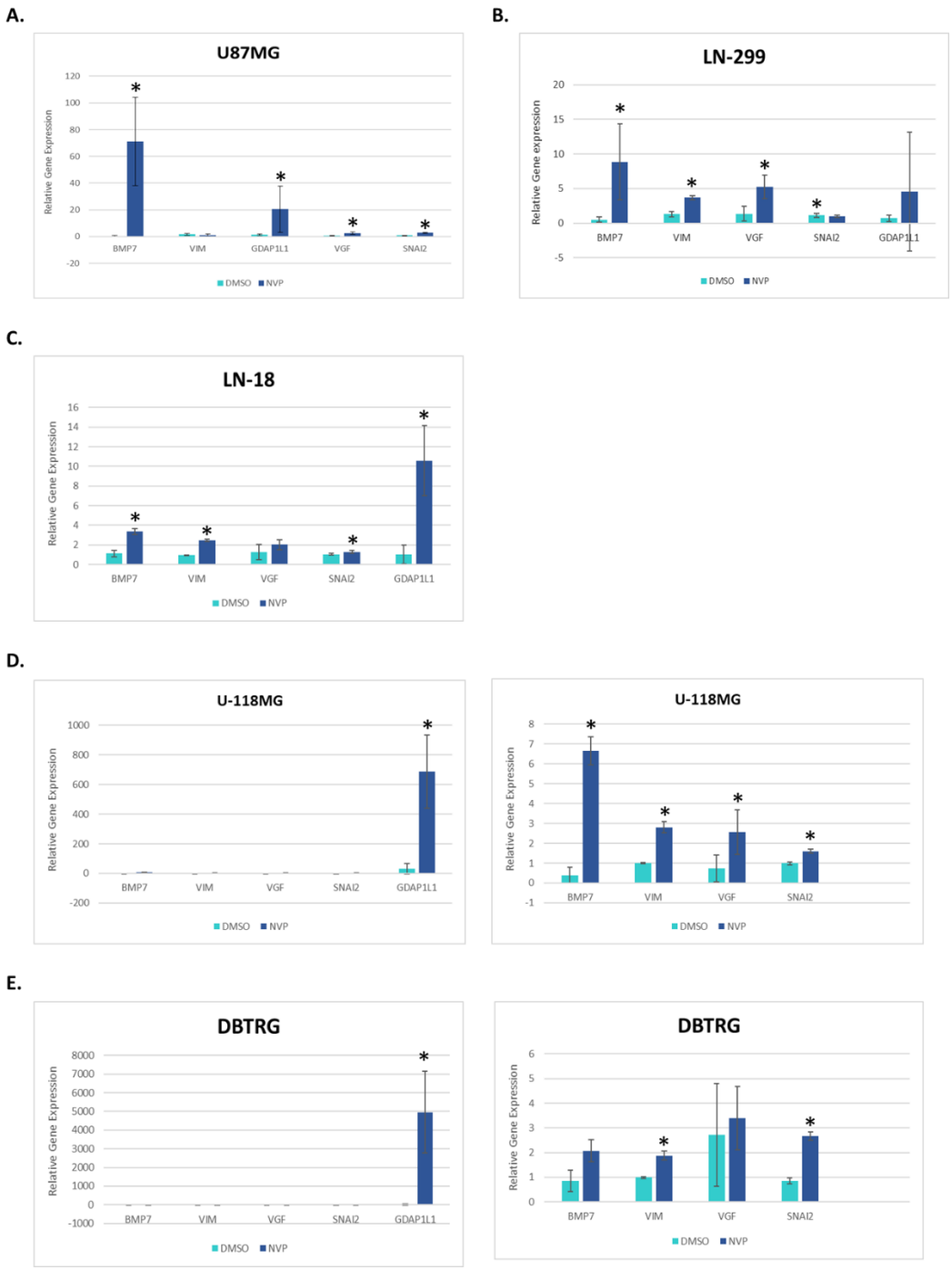

Figure 2: qRT-PCR validation of selected differentially expressed genes in multiple GBM cell lines. Relative gene expression of selected genes was analyzed in DMSO and NVP-*BEZ235* treated samples in the **(A)** U87MG, **(B)** LN-229, **(C)** LN-18, **(D)** U-118MG and **(E)** DBTRG cell lines. The error bars represent the standard deviation of the genes (n=5).

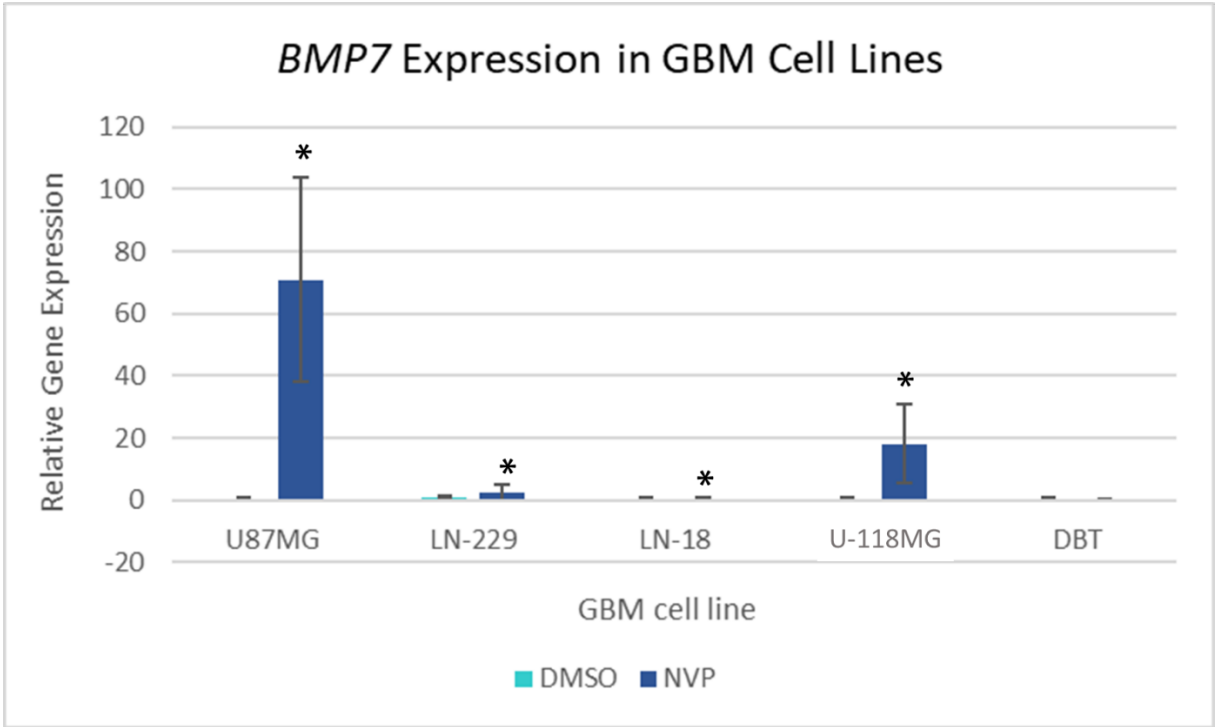

Figure 3: *BMP7* expression in multiple GBM cell lines. qRT-PCR was performed to analyze the relative gene expression of BMP7 in DMSO and NVP-*BEZ235* treated samples in the **(A)** U87MG, **(B)** LN-229, **(C)** LN-18, **(D)** U-118MG and **(E)** DBTRG cell lines. The error bars represent the standard deviation of the genes (n=5).

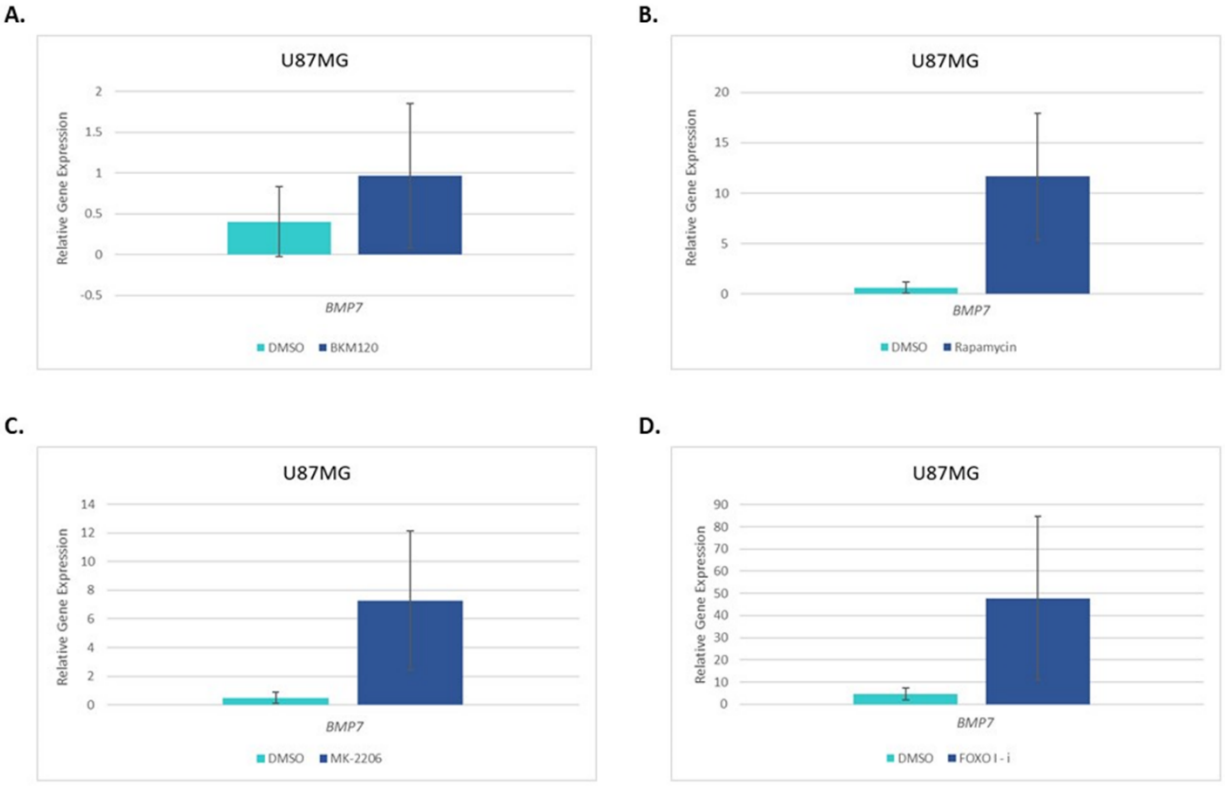

Figure 4: *BMP7* expression in BKM120-, Rapamycin-, MK-2206-, and FOXO 1-i- treated U87MG cells. qRT-PCR was performed to analyze the relative gene expression of *BMP7* in **(A)** BKM120-, **(B)** Rapamycin, **(C)** MK-2206-, and **(D)** AS1842856 (FOXO 1-i) -treated samples. The error bars represent the standard deviation of the genes (n=5)

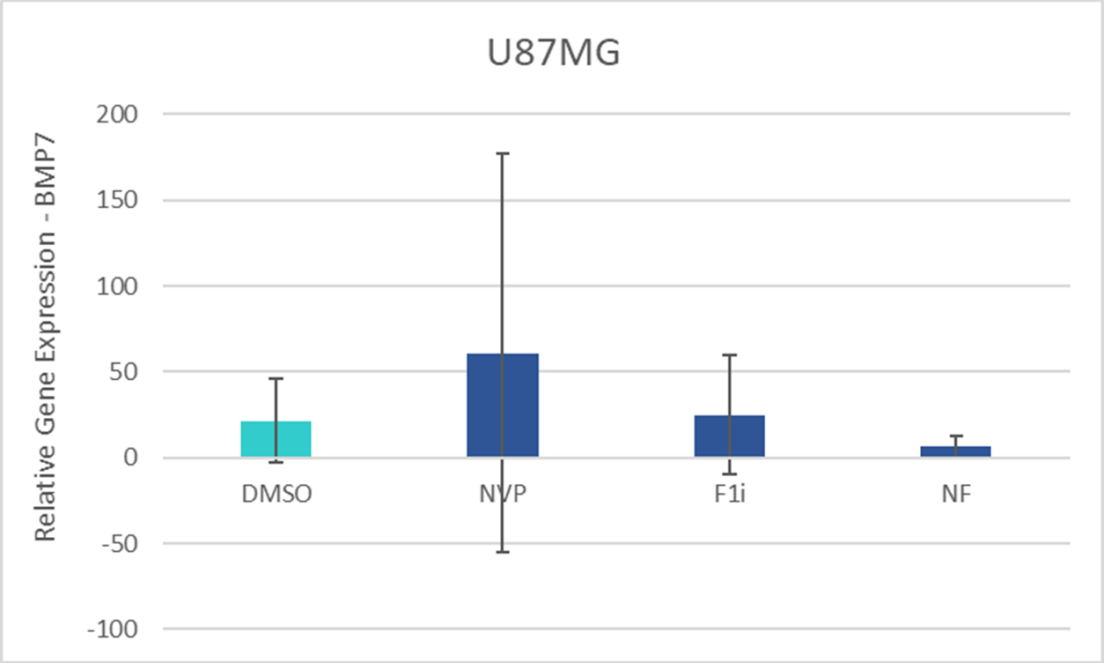

Figure 5: *BMP7* expression in NVP-*BEZ235*, FOXO 1-i and NVP-*BEZ235*/FOXO 1-i- treated U87MG cells. qRT-PCR was performed to analyze the relative gene expression of *BMP7* in **(A)** BKM120-, **(B)** Rapamycin-, **(C)** MK-2206-, and **(D)** FOXO 1-i-treated samples.
The error bars represent the standard deviation of the genes (n=5).

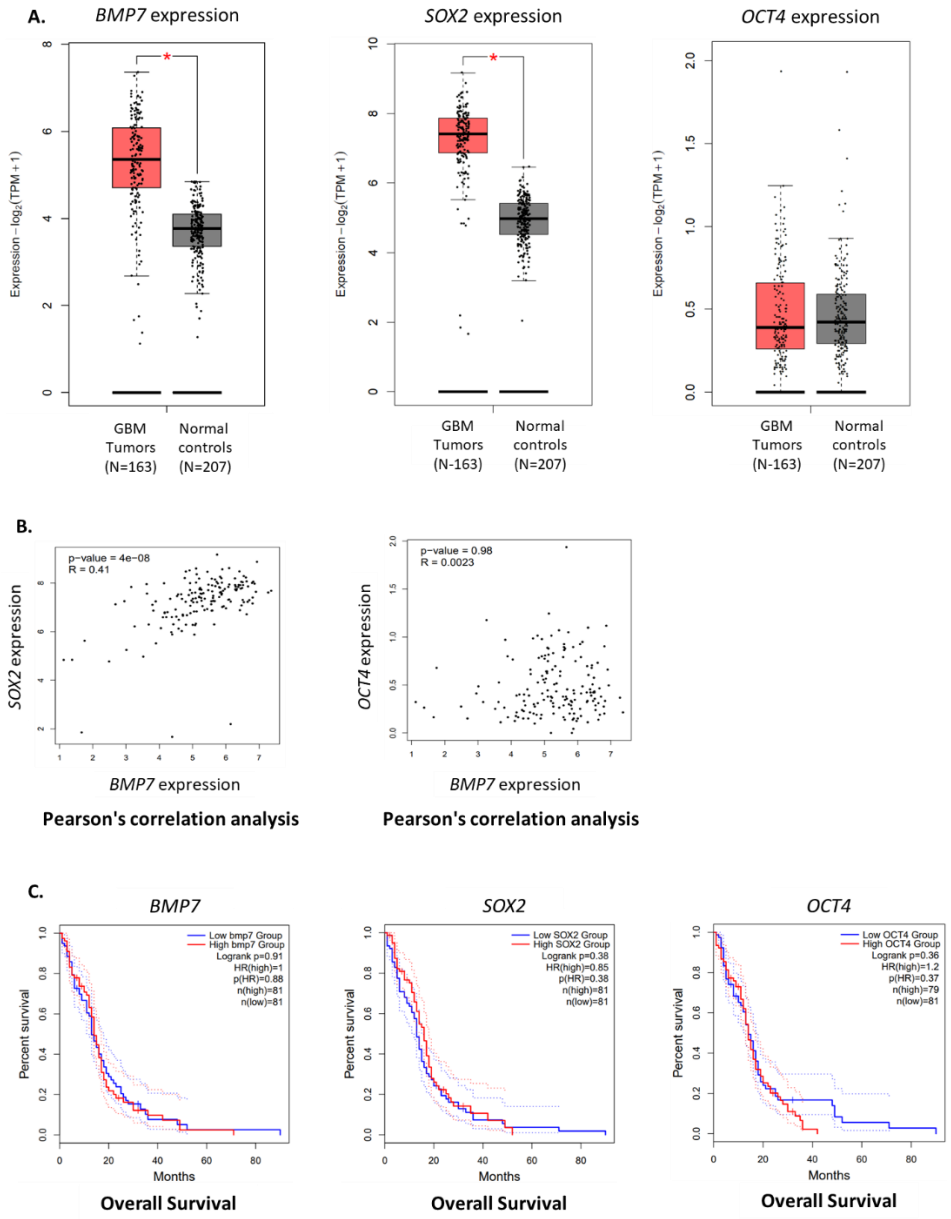

Figure 6: Expression of *BMP7*, FOXO 1-regulated genes *SOX2*, and *OCT4* in GBM tumors, and Pearson's correlation and overall survival (OS) analysis. Expression level analysis, and correlation and OS analysis data was retrieved from GEPIA2 online application based on information from The Cancer Genome Atlas (TCGA) database. **(A)** *BMP7* and *SOX2* expression levels were found to be up regulated in GBM tumors with respect to the normal controls. *OCT4* was not significantly expressed in GBM tumors. **(B)** Pearson's correlation analysis assessing *BMP7* correlation to *SOX2* and *OCT4*. *BMP7* and *SOX2* expression is correlated (p-value = < 0.05). *BMP7* and *SOX2* expression is not correlated (p-value = > 0.05).
(C) OS analysis shows the significant prognostic impact of *BMP7*, *SOX2*, and *OCT4* in GBM tumors.

BIOGRAPHICAL SKETCH

Yajaira Janett Macias was born in McAllen city in United States of America. She completed her primary school and high school in Mexico. Ms. Macias moved back to United Stated of America to continue her college education. She completed her Associate of Science in 2016 at South Texas College. Later, she completed her Bachelor of Science in 2018 at The University of Texas Rio Grande Valley. Ms. Macias entered the graduate program at The University of Texas Rio Grande Valley (UTRGV) in Fall 2019. She has earned a Master of Science (MS) in Biology in May 2021 from the UTRGV. She can be reached at yajaira.macias.rgv@gmail.com.