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EFFECTS OF LEPTIN ON ESTABLISHED GLIOBLASTOMA CELL LINES

Nicholas J. Cook
Northern Michigan University, nichcook@nmu.edu

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By

Nicholas Jon Cook

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This thesis by Nicholas J. Cook 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.

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Committee Chair: Robert Winn, Ph.D. Date

_____

First Reader: Robert Belton, Ph.D. Date

_____

Second Reader: Johnathan Lawrence, Ph.D. Date

_____

Third Reader: Richard Rovin, M.D. Date

_____

Department Head: John Rebers, Ph.D. Date

_____

Dr. Brian D. Cherry Date

Assistant Provost of Graduate Education and Research

ABSTRACT

EFFECTS OF LEPTIN ON ESTABLISHED GLIOBLASTOMA CELL LINES

By

Nicholas J. Cook

Glioblastoma is one of the most difficult cancers to treat because it is aggressive and resistant to therapy. The discovery of new therapeutic targets is drastically needed as zero improved treatment options have been added to the standard of care over the past 15 years. New and promising therapeutic targets are arising from psychosocial and environmental enrichment studies examining the role of stress in cancer progression. In animal models, eustress appears to slow tumor growth and recurrence resulting in increased overall survival and progression free survival while distress is associated with decreased overall survival. The cellular pathways activated by eustress were examined in glioblastoma cell lines; leading us to examine the use of β_3 adrenergic stimulation to decrease leptin gene expression as a possible novel therapeutic. The role of leptin and β_3 adrenergic stimulation were examined using a cell viability assay to assess changes in proliferation and quantitative PCR to assess gene expression. With the use of a selective β_3 agonist, leptin and leptin receptor mRNA was down regulated and resulted in decreased cell proliferation. Leptin's observed role in glioblastoma cell proliferation and survival was supported by treatments with a leptin antagonist, resulting in decreased cellular proliferation. This evidence would suggest further examination of leptin as a therapeutic target for glioblastoma.

DEDICATION

This thesis is dedicated to the patients and families of the Upper Michigan Brain Tumor Center. It is through their support that I was able to complete this work. The Upper Michigan Brain Tumor Center has provided the motivation to complete this endeavor and has shaped my future in the process. Two years ago during the Hope Starts Here Challenge Dr. Rovin shared the thought that eventually we may not need to have a fundraiser for the Upper Michigan Brain Tumor Center as we will find a CURE! I am confident that the Upper Michigan Brain Tumor Center will play a large role in that finding that cure.

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With all of the help and support I have received during this process from my time as a freshman fellow with the Upper Michigan Brain Tumor Center to now I cannot name all who have helped me or begin to express my gratitude. I would especially like to thank Dr. Robert Winn my graduate and undergraduate advisor and my research mentor Dr. John Lawrence for his help in designing experiments and helping with the statistical analysis from my freshman year on. I would also like to thank Dr. Richard Rovin, Dr. Robert Belton, and Cathy Bammert for their countless hours of support in and out of the laboratory. I would also like to thank the Upper Michigan Brain Tumor Centers amazing graduate students including Chris Steele, Ryan Passino, Ashley Parent, Keith Sabin, and Justin Segula. I would also like to thank the many undergraduates who helped in my research including Charlotte Cialek, Marissa Kane, Amer Mansoor, and Brian Carter. Most of all I would like to thank Elizabeth Wachowiak and my parents for their continued support during my time at Northern Michigan University and beyond.

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LIST OF SYMBOLS AND ABBREVIATIONS

β_3AR- Beta 3 adrenergic receptor

BCA- Bicinchoninic acid

BSA- Bovine serum albumin

bp- Base pair

CSC- Cancer stem cell

Ct- Cycle threshold

BSA- Bovine serum albumin

cDNA- Complementary deoxyribonucleic acid

Epi- Epinephrine

FBS- Fetal bovine serum

GBM- Glioblastoma multiforme

HSA- Hypothalamic-sympathoneural adipocyte axis

HSD- Honest significant difference

JAK – Janus kinase

MAPK – Mitogen-activated protein kinase

MDD- Major depressive disorder

mRNA- Messenger ribonucleic acid

PCR-Polymerase chain reaction

PI3K- Phosphoinositide 3-kinase

qPCR- Quantitative polymerase chain reaction

RLU- Relative light units

rt-PCR- Reverse transcriptase polymerase chain reaction

SHLA- Super human leptin antagonist

STAT3- Signal transducer and activator of transcription 3

VEGF- Vascular endothelial growth factor

INTRODUCTION

Many cancers have seen a vast improvement in progression-free survival or time without recurrence and prognosis in the last decade; the same is not true of glioblastoma multiforme (GBM). GBM is the most common and aggressive form of primary brain tumor. Each year 3.25 per 100,000 Americans will be diagnosed with GBM[1] with a progression free survival of often less than one year and statistics from 1995-2009 indicate a median one year prognosis of 35.7%[2]. The current standard of care for GBM is surgical resection and radiation followed by treatment with the chemotherapeutic agent temozolomide. The addition of temozolomide to the standard of care, nearly 15 years ago, increased the median survival from 12 months to 14.6 months[3]. Given the relatively poor prognosis, it is essential to investigate new forms of therapy.

Using an animal model to examine the relationship of stress and cancer, it was demonstrated that an enriched environment decreased the satiety hormone, leptin and significantly slowed cancerous growth, by increasing apoptosis and decreasing proliferation[4]. While leptin has been shown to be oncomodulatory in a variety of cancer types, leptin's role in glioblastoma is unknown[5]. However, leptin and leptin receptor are highly expressed in a majority of glioblastomas[6]. The objective of this work is to determine what effect leptin has on human glioblastoma cell lines and to examine the viability of anti-leptin therapies.

Chapter One: Literature review

Cancer and Development

One of the earliest changes noted in neoplastic tissue is enhanced receptor tyrosine kinase activity. This may be due to an increase in the number of receptors or constitutive activation. These tyrosine kinases often play a role in development but are being co-opted by cancer cells to promote proliferation and survival[7]. Evidence would suggest that leptin is playing a similar role. Leptin has been shown to be important as a growth factor in intrauterine development[8].

Leptin was first implicated in increasing the aggressiveness of cancer (oncomodulation) by studies reporting the correlation between obesity and the occurrence or poorer prognosis with cancer. Studies showing that when chronic stress, like that seen with depression, was alleviated patients benefited both in time before recurrence and overall survival[9] and some of this benefit may come from alteration in levels of leptin. Additionally, studies which implemented positive stress (eustress) as a treatment indicated a decrease in the rate of tumor growth by decreasing circulating leptin levels by increasing sympathetic nerve activation[4].

Stress and Cancer Progression

It has been shown that a diagnosis of cancer can lead to depression and depending on the type of cancer the range of patients suffering from major depressive disorder (MDD) may range from 2% to 49%[10]. Most of those patients will not seek treatment, it has been reported that the number of cancer patients experiencing major depression who seek treatment is only 10%[10]. Evidence suggests that stress, such as that seen with

depression, plays a role in poor prognosis as one meta-analysis found mortality rates are 39% higher in patients suffering from MDD[11].

While negative stress, such as that seen in depression may have negative effects on prognosis, positive stress or the alleviation of negative stress may result in better outcomes. For example the placebo used in clinical trials should have no clinical benefit, but in many cases, the control group of patients will have a better prognosis than their nonclinical trial cohort[12]. Additionally, in a study of 227 breast cancer patients enrolled in a support program designed to educate, reduce stress/anxiety and to form support groups the patients in the study saw increased progression free survival (2.8 years) compared to controls (2.2 years)[13]. Further, those whose cancer recurred lived longer with intervention (6.1 years) compared to control (4.8 years)[13]. By alleviating neurological depressive symptoms a significant clinical benefit was achieved, yet the exact physiological mechanism is unknown.

Environmental Enrichment

In animal models it has been shown that housing animals in enriched environments may decrease stress levels and provided significant clinical benefits[14]**.** Environmental enrichment has inhibited tumor formation in transgenic mice (APC +/-) which spontaneously develop colon cancer[4]. In addition Cao et al. found that environmental enrichment reduced the growth of orthotopically injected melanoma (B16 cell line) when, compared to controls[4]. Tumor mass was reduced by 77% at 6 weeks post injection with several mice not developing an observable tumor until necropsy[4]. The physiological effect of environmental enrichment that slowed tumor growth was increased apoptosis, decreased cell proliferation along with an increase in natural killer

cell and cytotoxic T cell activity[4]. Further, an enriched environment increased progression-free survival, compared to non-enriched controls in lymphoma vaccinated mice[15]. However, a recent study by Westwood attempting to replicate the impact of environmental enrichment on tumor growth found no statistical difference between standard housed mice and environmentally enriched mice but reported no molecular data[16].

Orthotopically injected mammary tumors in mice demonstrated that environmental enrichment increased apoptosis as measured by caspase 3 activity in environmental enriched mice versus control[16]. It was shown that when breast cancer cells (E0771) were treated with serum collected from an environmentally enriched mouse a 20% decreased proliferation occurred compared to serum collected from standard enriched mice after 24 hours of treatment[17].

By examining molecular changes it was determined that cancerous growth was inhibited by environmental enrichment through activation of the hypothalamic-sympathoneural adipocyte axis (HSA) described by Cao[4]. HSA pathway is initiated by increased expression of the immediate early gene brain derived neurotropic factor (BDNF), that in turn activates the sympathetic innervation of adipocytes via the β-adrenergic receptors leading to decreased leptin expression. Cao showed environmental enrichment on average decreased circulating leptin levels by 13% compared to control[4]. The normal role and oncomodulatory role of leptin will be discussed later in greater detail.

Adrenergic Signaling and Leptin in Adipose Tissue

While the HSA axis innervates adipocytes, the sympathetic branch of the autonomic nervous system can also effect the release of the catecholamines, epinephrine and norepinephrine. Epinephrine is released by the adrenal glands when stimulated during a stress response which is most commonly caused by an emotional response[18] or exercise[19]. A multitude of adrenergic receptors (α and β) exists to which epinephrine can bind causing varied effects, either stimulatory or inhibitory. These receptors are widespread throughout the body with different tissue types being predisposed to expressing different types of adrenergic receptors. While β_1 and β_2 adrenergic receptors are wide spread throughout the body β_3 adrenergic receptors are limited in their distribution to bladder, colon, and brown adipose tissue in humans[20].

When comparing obese animals to healthy weight animals, the β_3 adrenergic receptor (β_3AR), a thermogenic receptor in brown adipose tissue which helps maintain body temperature, was found to be down regulated in the obese animal in brown adipose tissue[21]. The infusion of epinephrine into human subjects has been shown to decrease circulating leptin levels[22]. A study by Getty aimed at determining which β adrenergic receptor subtype (1-3) inhibited leptin release reported that β_3 adrenergic receptor activation inhibited leptin secretion but found no inhibition with activating β_1 and β_2 adrenergic receptors [23]. Comparing rodent studies to humans, rodents express high amounts β_3AR on white adipose tissue while humans primarily have β_3 adrenergic receptors on brown adipose and not white adipose[24].

Leptin

Leptin acts to signal satiety as it acts as an indicator of fat stores in the central nervous system and modulates energy balance and feeding behavior through acting on the hypothalamus[25]. Leptin was discovered by Zhang who mapped out the mutation responsible for creating phenotypically obese mice (ob/ob mice) which lacked leptin[26]. When supplemented with exogenous leptin these ob/ob mice returned to a normal weight[26]. Another type of obese mouse named db/db lack a functional leptin receptor resulting in increased leptin levels[27]. Yet, the majority of obesity in humans does not appear to be caused by a lack of leptin or dysfunctional leptin receptors. Instead, obese individuals have high levels of adipose tissue; the main source of leptin, which results in elevated serum levels of leptin and decreased sensitivity[28]. A link to leptin and cancer was postulated when obesity was found to be strongly associated with a greater risk of developing cancer[29,30].

Leptin's Oncomodulatory Role

While systemic leptin can act on cancerous growth, leptin also acts locally as a paracrine factor secreted by several tumor types and binds to leptin receptors on adjacent cancerous cells being independent of systemic leptin. The overexpression of leptin (or its receptor) occurs in many cancers including ovarian[31], colorectal[32] and breast cancer[33] but leptin is not found in surrounding tissue.

Leptin has been shown to be oncomodulatory or increase the invasive and aggressive abilities in colon cancer[34], prostate cancer[35], neuroblastoma[36], and glioblastoma[5]. In brain tumors, leptin and leptin receptor are overexpressed but not expressed in normal brain tissue and in lower grade tumors[6]. In GBM and anaplastic

astrocytoma 80.5% of 87 samples from Riolfi's study were positive for leptin and its receptor while lower grade brain tumors (gangliogliomas and grade 1 and 2 astrocytomas) had lower levels of expression of leptin and its receptor[6]. This study reported a significant correlation between leptin and leptin receptor with the degree of brain tumor malignancy[6].

The molecular basis for leptin's oncomodulatory effect is attributed to activation of proliferative and anti-apoptotic pathways. It was demonstrated that decreasing leptin expression by 50% using siRNA increased apoptosis of rat C6 GBM cells in vitro[37]. The MAPK and JAK/STAT pathway are known to be activated by leptin to mediate proliferation[38,39] (Figure 1). Leptin activates the PI3K pathway to protect against apoptosis[40]. Little is known about leptin's role in GBM in humans. While the direct actions of leptin in GBM is unclear, one of the downstream signaling pathways of the leptin receptor (STAT3) when inhibited by siRNA significantly decreased cell proliferation in human GBM cells[41].

Leptin has also been shown to act as an independent angiogenic factor via the PI3K and MAPK pathways, as well as acting synergistically to increase the expression of vascular endothelium growth factor (VEGF)[42]. Activated VEGF receptor also acts through the same signaling pathways as leptin receptor activation (PI3K and MAPK) for its effect[43]. Both VEGF and leptin are upregulated during hypoxia which is of importance as the microenvironment of GBM can be extremely hypoxic with as little as 0.1% oxygen. Increased hypoxia was been associated with increased aggressiveness of the tumor[44]. Angiogenesis is mediated by increases in hypoxia inducible factors (HIF) 1 and 2. Of these two subfamilies of HIF, HIF 2 increases during chronic hypoxia and HIF 1 increases during acute hypoxia, it is HIF 1 that plays a larger role in angiogenesis. HIF 1

effects leptin and VEGF gene expression through a hypoxia response element (HRE) in leptin's promoter region[45]. Hypoxia driven leptin expression has been described in colorectal cancer as well as in adipose tissue[34].

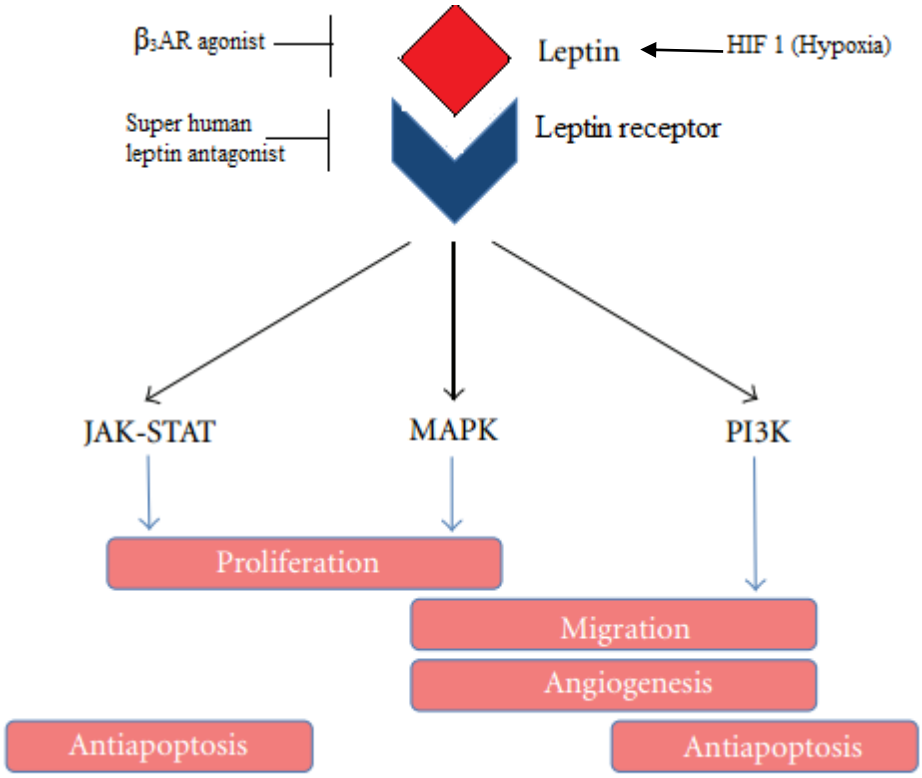

Figure 1: Cell signaling pathways co-opted by cancer through leptin receptor (ObR) activation[5]. Pathways include the Janus Kinase (JAK), Signal Transducer and Activator of Transcription 3 (STAT) and the Mitogen Activated Protein Kinase (MAPK) signaling cascade both of which lead to increased cell proliferation. The MAPK and Phosphoinositide 3-kinase (PI3K) signaling pathway both lead to increased invasiveness by enhancing migration and angiogenesis or vascularization of the tumor. Also the PI3K and JAK-STAT pathway provide apoptosis resistance. Inhibition of leptin includes Super human leptin antagonist which inhibits leptin from binding to its receptor and β_3AR agonist which down regulated leptin and leptin receptor gene expression while hypoxia through HIF 1 increase leptin gene expression.

Leptin and Drug Interactions

Leptin appears to have a role in providing resistance to various cancer treatments coupled to its oncomodulatory role. Leptin has been found to inhibit the efficiency of the antiestrogen ICI 182,780 in breast cancer, possibly through the activation of the JAK-STAT pathway[46]. These effects support clinical data that obesity (increased circulating leptin) increases the resistance to antiestrogen therapy[46]. Additionally, leptin has been shown to decrease the cytotoxicity of fluorouracil (5-FU) in colon cancer, although the specific mechanism is unknown, and may be due to the apoptotic resistance leptin provides[47]. Bevacizumab is an anti-VEGF treatment used in colon cancer and it has been demonstrated that a greater proportion of visceral fat has been correlated with poorer prognosis[48,49]. In GBM, Bevacizumab provides a minimal change in progression free survival at 6 months from 32% to 42%[50]. As VEGF is inhibited from binding, leptin remains present. Heightened acute hypoxia may drive the expression of leptin which can then act independently as an angiogenic factor and the tumor will still become vascularized[51]. As leptin plays an oncomodulatory role in many cancer and is highly prevalent in GBM providing resistance to current therapies it is essential to investigate leptin's role in GBM.

Project Objectives

With mounting evidence that leptin increases GBM aggressiveness and provides resistance to treatment, coupled with evidence that GBM overexpresses leptin, it is essential to further examine leptin's effects in GBM. The effect of β_3AR activation on GBM cells will be examined in this paper. In adipose tissue, stimulating β_3 adrenergic receptors results in decreased leptin secretion and it is known that, in some cancers, β_3AR is unregulated, however the status of β_3 adrenergic receptor is unknown in GBM. We

hypothesize that a β_3AR agonist will decrease leptin gene expression and decrease glioblastoma cell proliferation. Additionally, we anticipate that use of a high affinity leptin receptor antagonist, to inhibit leptin from binding to leptin receptor, will also decrease GBM cell proliferation and provide additional evidence of leptin's role in GBM.

Chapter Two: Experimental Design

Introduction

While leptin has been found to be overexpressed in a high percentage of GBM patient samples it is unknown whether established GBM cell lines (T98, LN229) express leptin and leptin receptor. β_3AR agonists are a potent inhibitor of leptin in adipocytes. While it is known that β_3ARs are upregulated in colon cancer the expression status of β_3AR and their role in leptin regulation of GBM cell lines is currently unknown. Amplification of mRNA for leptin and leptin receptor will be used to determine the presence of leptin in established GBM cells lines. The appearance of mRNA for β_3 adrenergic receptor will provide a possible treatment option to target leptin expression.

GBM Cell Lines

T98, LN229, and U87 GBM cell lines (American Tissue Culture Collection Manassas, VA) were cultured in standard conditions of 37°C and 5% CO_2 in complete media, Eagle's minimal essential medium (Lonza, Walkersville, MD) with 10% fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA) and 1% Penicillin/Streptomycin/Amphotericin B (Gibco, Carlsbad, CA)) until over 75% confluent in a T-25 flask. Cells were removed by trypsin-versene (Lonza) and pelleted at 300x g.

RNA Extraction

RNA was extracted using an RNAeasy kit (Qiagen, Germantown, MD) following the recommended protocol. Briefly, the supernatant was removed and the pelleted cells were suspended in 350 µl RLT buffer and vortexed. Cell lysate was then homogenized using centrifugation using the QIA Shredder spin column (Qiagen) following the manufacturer's published protocol. RNA was then isolated from the cell lysate and stored at -20°C and converted to cDNA within 7 days.

Reverse Transcriptase PCR

RNA was converted to cDNA using the AMV reverse transcriptase kit (Promega, Madison, WI) following the manufacturer's protocol. First 2 µl of RNA was added to a 0.5 ml thin-walled PCR reaction tube along with 1 µl of random hexamer primers and heated to 70°C for five minutes then cooled on ice. Once cooled, the following components were added to the reaction: 5 µl of AMV Reverse Transcriptase 5X Reaction buffer, 2.5 µl of dNTP mix, 40 units of RNasin Ribonuclease Inhibitor, and 30 units of AMV reverse transcriptase (enzyme). The reaction was incubated at 37°C for 60 minutes, cDNA was stored at -20°C until further use. The cDNA was used for PCR amplification for the following genes: leptin ligand, leptin receptor, β_3 adrenergic receptor and GAPDH (housekeeping gene). PCR reactions (25 µl) were set up according to manufacturer's suggested protocol using 2x PCR Master Mix (Promega) (12.5 µl), forward and reverse primer set (1 µl each), cDNA template (5 µl), and nuclease free water (5.5 µl). PCR amplification was performed using an initial denaturation step at 95°C for 2 minutes, followed by 30 cycles: denaturation at 95°C for 30 seconds, annealing at 55°C for 30

seconds, and extension at 72°C for 30 seconds with a final extension at 72°C for 5 minutes. PCR products were examined by running on a 1.2% agarose gel containing 0.5 µg/ml ethidium bromide at 70 volts for 45 minutes in 1x (Tris-Borate EDTA, TBE, buffer). Products were visualized under ultraviolet light using a Molecular Imager Gel Doc (BioRAD, Hercules, CA).

Designing PCR Primers

Primer sets were designed for real time PCR using primer 3 on My Biology Workbench (http://workbench.sdsc.edu/). Then primer-BLAST was used to ensure specificity to target genes without the amplification of off-target mammalian homologues and IDT Oligo analyzer tools (http://www.idtdna.com) were used to ensure favorable annealing to target sequence rather than primer sets forming dimers.

Primer Sets (Human)

Table 1: IDT Primer sets used for amplification of human cDNA for reverse transcriptase PCR (RT PCR) and quantitative PCR (qPCR). DNA sequences shown in 5' – 3' orientation.

Primer (Human)	Sequence 5' - 3'
Beta Actin Forward	AAG GTG TGG TGC CAG ATT TTC
Beta Actin Reverse	TCG ACA ACG GCT CCG GCA T
Beta-3 Adrenergic Receptor Forward	TGA AAT CCA GTT GCC ATT GA
Beta-3 Adrenergic Receptor Reverse	ATT TGA CCA ACC CAA CA AT
qPCR GAPDH Forward	AGA GGG AGG GAT GAT GTT
qPCR GAPDH Reverse	TGC ACC ACC AAC TGC TTA
RTPCR GAPDH Forward	AAC TTT GGC ATT GTG AAG GGC TC
RTPCR GAPDH Reverse	TGG AAG AGT GGG AGT TGC TGT TGA
Leptin Forward	GAA GAC CAC ATC CAC ACA CG
Leptin Reverse	AGC TCA GCC AGA CCC ATC TA
Leptin Receptor (V1-3) Forward	TGG GTC TTC GTA TGA GGT TCA GGT
Leptin Receptor (V1-3) Reverse	GCT TTC CGC AAG TGA CTG GAT TGT

Cell Proliferation Assay

Introduction

To determine the effects of leptin on GBM proliferation using the T98 and LN229 cell lines, exogenous leptin was added to cell culture medium. To further investigate leptin's role in proliferation of T98 and LN229 cells, anti-leptin therapies were used *in vitro*. These treatments included β_3AR agonists (Sigma, St. Louis, MO) and Super Human Leptin Antagonist (Protein Laboratories Rehovot, Israel) to examine the effects of endogenous leptin. Endogenous leptin was expected to be depleted by down regulating the expression of leptin and leptin receptor via β_3AR agonist stimulations. Blocking leptin from binding to its receptor with the Super Human Leptin Antagonist, the direct effects of endogenous leptin were further observed.

Treatment of GBM Cell Lines

T98 and LN229 cells were grown in standard conditions until 75% confluent at which time cells were removed by trypsin and pelleted at 300x g and were suspended in complete media. Cells were then counted using a hemocytometer and plated at 5,000 cells in 100 µl per well in a 96 well plate (at least 6 wells for each treatment group) in complete media. Cells were allowed to adhere to the plate for 24 hours before media was removed and treatment added. Treatments consisted of Epinephrine (0.025 ng/ml-2.25 ng/ml), β_3 Adrenergic receptor agonist, BRL 37344, (400 ng/ml), recombinant leptin (200 ng/ml), Super Human Leptin Antagonist, (200 ng/ml) and complete media as a control. Super human leptin required a vehicle control of 1% bovine serum albumin (BSA). Experiments were performed in triplicate or greater. Treatments were at physiologically relevant serum concentrations. Reported baseline serum levels of epinephrine is 0.05 ng/ml, serum epinephrine levels have been reported to be 0.160 ng/ml for mild stressors

like mental arithmetic[52] and 0.4 ng/ml in higher stress situations as measured during an oral presentation at a medical conference[18].

Quantifying Cell Proliferation

To quantify the number of viable cells 100 µl of Cell Titer-Glo Luminescent Cell Viability Assay (Promega) was added to each well according to the manufacture's protocol. After incubating at room temperature for 30 minutes luminescence was measured using a Modulus Microplate Reader (Promega). Cell Titer-Glo measures viable cells by quantifying the relative amount of ATP. Experimental groups were reported as change of relative light units (ΔRLU) from controls.

Quantitative PCR

RNA was obtained from 3 wells of a 96 well plate by removing the media and adding 112.5 µl of RLT buffer to each well. Cell lysate was homogenized as previously described and RNA extracted by RNeasy kit as previously described. RNA was converted to cDNA by AMV reverse transcriptase as previously described. Quantitative PCR was performed using Sybr Green Master Mix (Life Technologies, Carlsbad, CA) and following the manufacture's protocol. Each reaction in a 48 well reaction plate (20 µl total) contained 2x Sybr Green Master Mix (10 µl), Forward and Reverse Primer (1 µl each of 10 nM primer), cDNA template (2 µl), and nuclease free water (6 µl in reactions with cDNA template and 8 µl in negative control wells). Reactions were performed in a StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA) according to Sybr Green Master Mix cycling protocols; 10 minute incubation at 95°C for Taq polymerase activation, 15 second denaturation at 95°C , 60 second annealing step at 55°C, and 60 second extension at 72°C for 40 cycles. Change in relative gene expression was

compared using the 2^-ΔΔCT method as each PCR cycle difference should be a two^{CT} change in gene expression[53].

Statistical Analysis

Data was analyzed using SPSS (Microsoft, Redmond, WA). Epinephrine and leptin data and leptin and β_3AR agonist data were analyzed by one-way ANOVA. Tukey's HSD was used as a post-hoc analysis to determine significant differences. A paired t-test was used to analyze qPCR data on the ΔCT between untreated and β_3AR agonist for leptin ligand and leptin receptor in T98 and LN229 cell lines. Analysis of β_3AR agonist and SHLA in LN229 cell line was conducted using a one sample t-test compared to untreated control. Interassay variability was assessed dividing the standard deviation of the treatment averages by the overall average, with an acceptable range of less than 20% variability between similar treatments. Intraassay variability was assessed by dividing the standard deviation of the sample by the average, with an acceptable range of less than 10% variability.

CHAPTER THREE: RESULTS

Identification of Leptin, Leptin Receptor and β_3AR in GBM Cell Lines

In T98, LN229, and U87 GBM cell lines, mRNA for leptin receptor, leptin ligand, β_3AR, and GAPDH was amplified by rt-PCR (Figure 2). Amplification bands are easily observable for β_3AR in T98, LN229, and U87 GBM cell lines. A faint band is present for leptin receptor in T98 and LN229 cell lines. A band for leptin was easily observable in

both T98 and LN229. Quantitative PCR amplified leptin and leptin receptor for comparison (Figure 6).

Treatment with Epinephrine and Leptin is Not Additive

Examining the role of stress in GBM with the use of epinephrine provided unexpected results. We asked the initial question of what effects sympathetic activation of adrenergic receptors would have on GBM cells in culture. Epinephrine has been shown to possess a broad range of effects acting upon multiple adrenergic receptors. Physiologically relevant levels of epinephrine (0.3 ng/ml to 0.5 ng/ml) observed in serum during a stress response as reported in the literature[18,52] resulted in increased proliferation. Cells treated with epinephrine proliferated with both basal (0.025 ng/ml to 0.075 ng/ml) and high stress levels (0.3 ng/ml to 0.5 ng/ml) of epinephrine increased RLU approximately 10% from untreated cells (Figure 3). T98 cells treated with leptin showed a slight overall increase in growth at low levels (200ng/ml) compared to a negative change from control at higher concentrations (400 ng/ml) both of which were not significantly different from control. However epinephrine at basal and stress levels in addition to leptin significantly decreased proliferation as measured by a 6-7% negative change in RLU from control.

A one-way ANOVA was computed using SPSS to compare changes in relative light units from a cell viability assay. Six different treatment groups were compared: leptin treatment at 200 ng/ml (treatment 1), leptin treatment at 400 ng/ml (treatment 2), epinephrine at basal levels, 0.025 – 0.075 ng/ml, (treatment 3), epinephrine at high stress

levels, 0.3 -0.5 ng/ml, (treatment 4), leptin 200 ng/ml in conjunction with basal levels of epinephrine (treatment 5), and leptin 200 ng/ml in conjunction with high stress levels of epinephrine (treatment 6). A significant difference was found among treatments ($F(5,245) = 13.959$, $p<0.000$). Tukey's HSD was used to determine the nature of the differences between treatments. This analysis revealed that change from the control was highest in high stress epinephrine treatment (m = 10.23%, sd = 9.542%) and was significantly different from that of leptin 200 ng/ml (treatment 1: m = 1.81%, sd = 12.332%) and leptin 400 ng/ml (treatment 2: m = -2.92%, sd = 18.485%). High stress epinephrine treatment was also significantly different from leptin and basal levels of epinephrine (treatment 5: m = -6.89%, sd = 16.162%) and significantly different from high stress epinephrine and leptin treatment (treatment 6: m = -7.34%, sd = 12.647). However, high stress epinephrine was not significantly different from basal levels of epinephrine treatment (treatment 3: m = 9.66%, sd = 8.22%). Basal epinephrine treatment was significantly different from both leptin treatments and leptin in combination of high and basal levels of epinephrine. Leptin treatment at 200 ng/ml was significantly different from treatment with leptin in combination with high and basal levels of epinephrine.

Upon identifying leptin, leptin receptor, and β_3AR mRNA in GBM cell lines and moderate response to epinephrine T98 and LN229 cell lines were treated with BRL37344 a selective β_3AR agonist to see what effects activating only the β_3AR would have on cell growth.

β_3AR Agonist Effects Cell Proliferation

Treating the GBM cell line T98 with BRL37344 decreased cell proliferation approximately 10% (Figure 4). When treated with 200 ng/ml of leptin GBM cells

proliferated at a rate similar to untreated cells. However, when cells were treated with leptin combined with a BRL37344 cell viability decreased by 9.5% on average (0% - 9%). Intraassay variability was low in each plate (3.6% - 7.4%); however interassay variability was high 26% - 30%. A one-way ANOVA was computed using SPSS comparing change in relative light units in three different treatment groups; leptin treatment, leptin in conjunction with BRL37344, and BRL37344 alone. A significant difference was found among treatments ($F(2,249) = 8.643$, $p<0.000$). Tukey's HSD was used to determine the nature of the differences between treatments. This analysis revealed that change from control was the least in leptin treatment (m = -4%, sd = 12.055%) and was significantly different from leptin/ BRL37344 agonist (treatment 2: m = -9.53, sd = 10.39%) and BRL37344 agonist alone (treatment 3: m = -9.55, sd = 8.316%). However, the combination of leptin and BRL37344 agonist was not significantly different from the BRL37344 agonist treatment alone.

BRL37344 treatment alone deceased RLU by 10% in T98 and 15% in LN229 cell lines compared to control (Figure 9). A one sample t-test comparing the mean change in RLU of the BRL37344 treated groups to control found a significant difference between the means of each group and control. LN229 treated with β_3AR agonist ($t(23) = -6.374$, $p < 0.001$) with a sample mean of -15.44% (sd = .11866) was significantly less than the control. T98 treated with BRL37344 ($t(83) = -10.979$, $p < 0.001$) with a sample mean of -9.96% (sd = 0.08316).

β_3AR Treatment and Leptin Expression

Using qPCR on a subset of cells treated with a BRL37344 in T98 and LN229 cell lines expression of leptin and leptin receptor decreased (Figure 6). Gene expression levels

of untreated cells were compared with those of cells treated with 400 ng/ml of BRL 37344. The collated data from six qPCR runs were as follows; the relative expression of leptin was reduced by 3%-34% when compared to controls, T98 leptin expression decreased to 97% of control, (range: 0.84 -1.12) p= 0.278 with overlap between standard error for treated and untreated and LN229 leptin expression decreased to 66% of control (range: 0.64- 0.68) p< 0.01. The relative expression of leptin receptor was reduced by 36%-52% when compared to controls with T98 leptin receptor expression reduced to 48% of control (range: 0.36 -0.63) p=0.05 and LN229 leptin receptor expression reduced to 64% (range: 0.58- 0.72) p= 0.0594. Significance determined using a paired (one tailed) t=test between BRL37344 treated and control ΔCT.

Quantitative PCR on GBM cell lines T98, U87, and LN229 was used to compare the relative expression of leptin using beta actin as a house keeping gene (Figure 7). LN229 had the highest proportion of leptin arbitrarily set at 100% (range: 0.7 – 1.42) and U87 leptin levels at 85% (range: 0.61 – 1.2) and T98 leptin levels being the lowest relative amount 57% (range: 0.47 – 0.65).

Super Human Leptin Antagonist

Treating LN229 cell line with SHLA and BRL37344 as a negative control yielded decreased proliferation as measured by ΔRLU. SHLA decreased ΔRLU by 15.58% ($p < 0.001$) on average while β3AR agonist decreased ΔRLU by 15.44% ($p < 0.001$) on average (Figure 8). Again intraassay variability was low ranging from 2.9% - 11.6%; however interassay variability was high 21.5% - 32.8%. A one sample t test comparing the mean change in RLU of the experimental to control groups found a significant difference between the means of both SHLA and BRL37344 and control. BRL37344

($t(23) = -6.374$, $p < 0.001$) with a sample mean of -15.44% (sd = .11866) was significantly less than the control, super human leptin antagonist ($t(29) = -13.562$, $p < 0.001$) with a sample mean of -15.58% (sd = 0.06292).

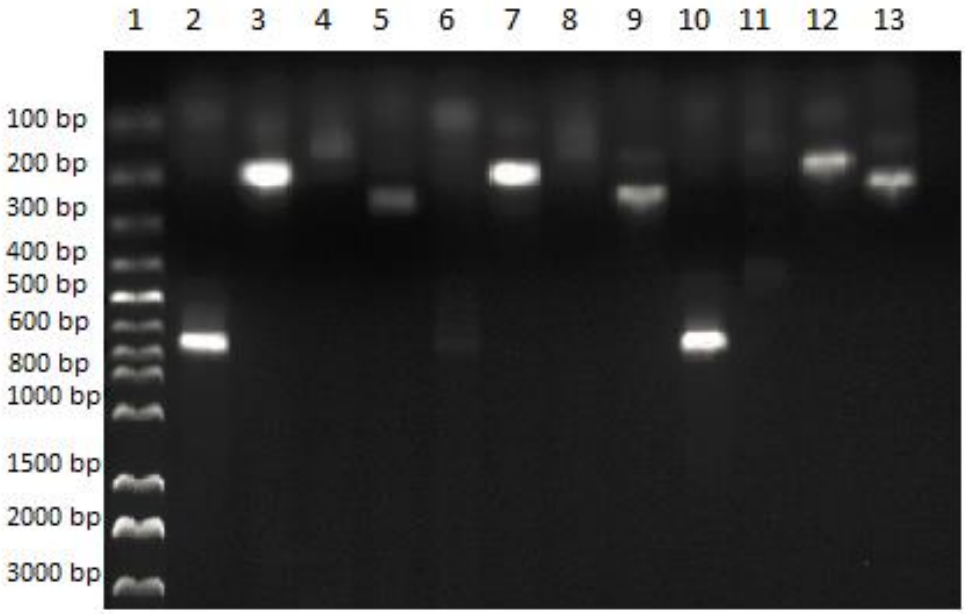

Figure 2: Identification of Leptin, Leptin Receptor and β_3AR mRNA in GBM cell lines LN229, T98, and U87. Lane 1: 100 bp ladder, Lane 2: GAPDH-LN229, Lane 3: Leptin-LN229, Lane 4: Leptin Receptor-LN229, Lane 5: β_3AR-LN229, Lane 6: GAPDH-T98, Lane 7: Leptin-T98, Lane 8: Leptin Receptor-T98, Lane 9: β_3AR-T98, Lane 10: GAPDH-U87, Lane 11: Leptin-U87, Lane 12: Leptin Receptor-U87, Lane 13: β_3AR-U87.

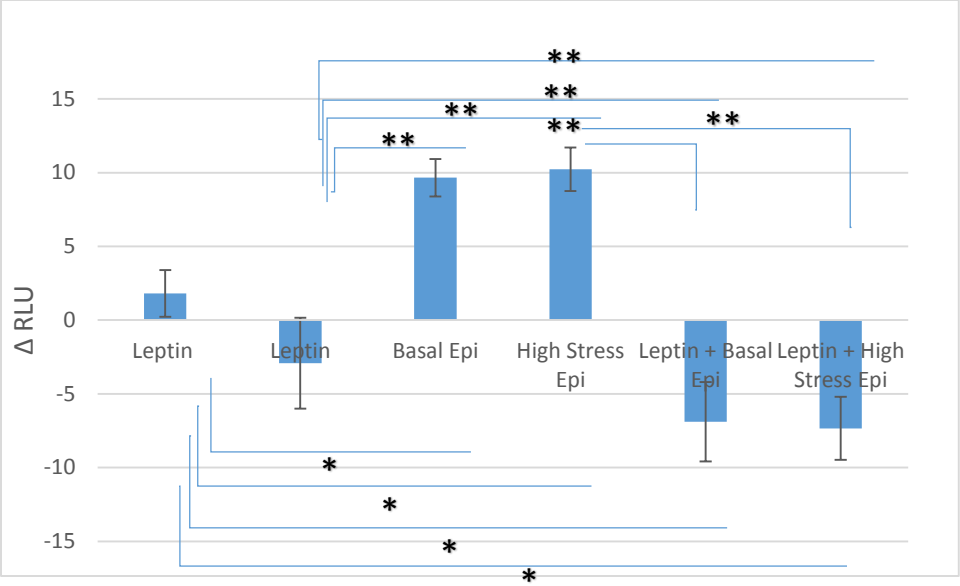

Figure 3: Effect of leptin and epinephrine on proliferation of T98 GBM cell line. Cells were plated at a density of 5,000 cells per well and allowed to adhere over 24 hours. Media was removed and cells were treated with 200 ng/ml and 400 ng/ml of leptin, 0.025 ng/ml -0.075 ng/ml of epinephrine (basal serum levels), 0.3 – 0.5 ng/ml of epinephrine (high stress serum levels), leptin in combination with basal levels of epinephrine and leptin in combination with high stress levels of epinephrine. After 48 hours of treatment relative cell change was determined using Cell Titer-Glo using change in relative light units (Δ RLU) from control as determined by a Modulus Microplate Reader. Significance shown by single asterisk (*) represents $p < 0.05$ double asterisk (**) represents $p < 0.01$.

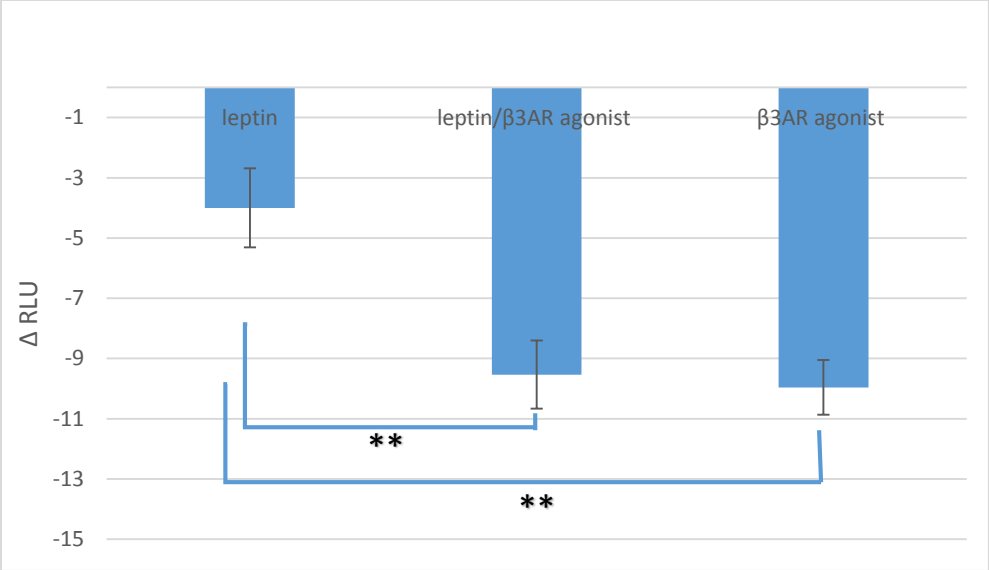

Figure 4: Effect of β_3AR agonist on proliferation of T98 GBM cell line. Cells were plated at a density of 5,000 cells per well and allowed to adhere over 24 hours. Media was removed and cells were treated with 200 ng/ml of leptin, 200 ng/ml of leptin and 400 ng/ml of BRL37344 and 400 ng/ml of BRL 37344 alone. After 48 hours of treatment relative cell change was determined using Cell Titer-Glo using change in relative light units (Δ RLU) from control as determined by a Modulus Microplate Reader. Significance shown by double asterisk (**) represents $p < 0.01$.

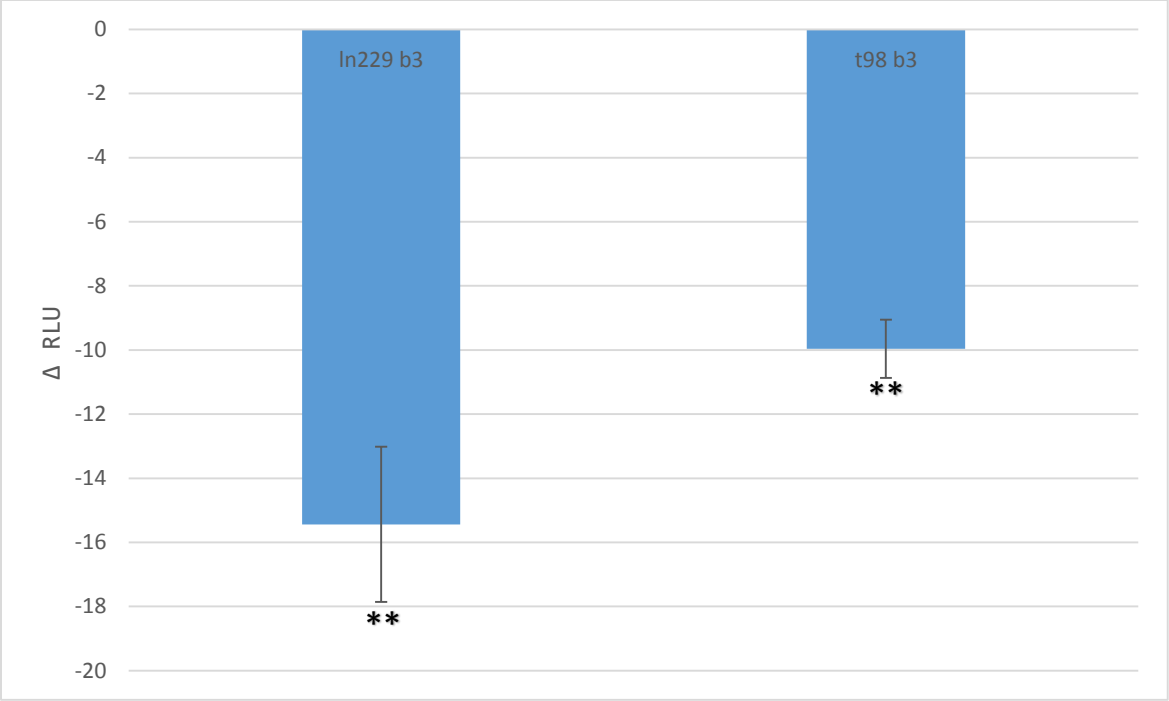

Figure 5: Effect of β_3AR agonist on proliferation of T98 and LN229 GBM cell lines. T98 and LN 229 GBM cell lines plated at a density of 5,000 cells per well in 12 wells and allowed to adhere over 24 hours. Media was removed and cells were treated with 400 ng/ml of BRL37344 (β-3 adrenergic agonist). After 48 hours of treatment relative cell number was determined using Cell Titer-Glo using relative light units as determined by a Modulus Microplate Reader. Δ RLU (the change in relative light units) was measured as compared to control. Analysis includes 4 separate 96 well plates. Three wells from each treatment groups were used to obtain mRNA to quantify relative expression levels of leptin and leptin receptor. Significance shown by single asterisk (*) represents $p < 0.05$ double asterisk (**) represents $p < 0.01$.

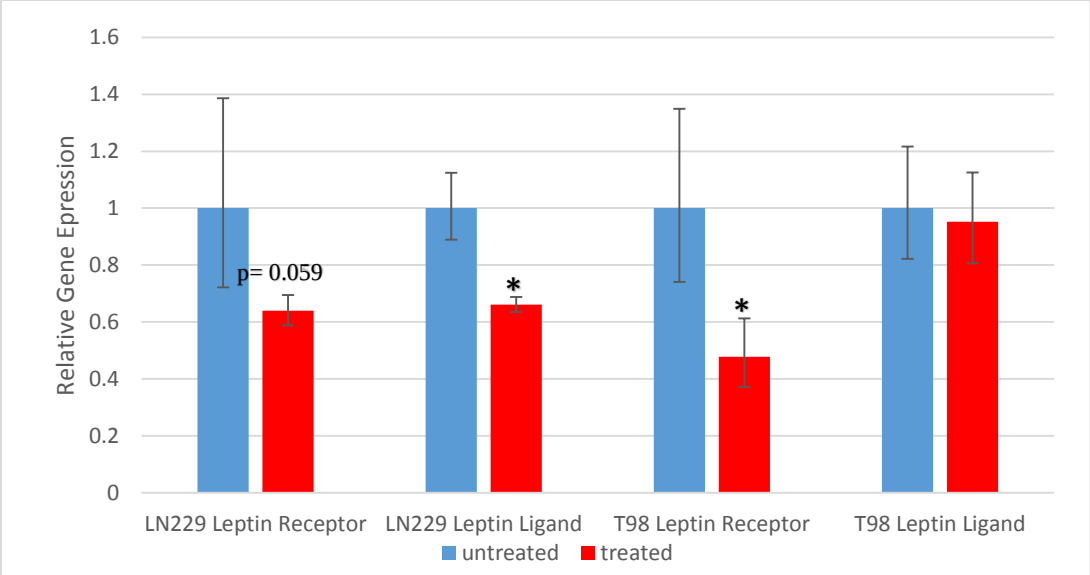

Figure 6: Relative gene expression of leptin receptor and leptin ligand in LN229 and T98 GBM cell lines treated with BRL37344. Relative gene expression normalized using GAPDH using the $2^{-\Delta\Delta CT}$ method. Change in gene expression from untreated cells (blue) of leptin ligand and leptin receptor with treatment of BRL37344 (β_3AR agonist) (red) in LN229 and T98 GBM cell lines. Significance from control shown by single asterisk (*) represents $p \leq 0.05$.

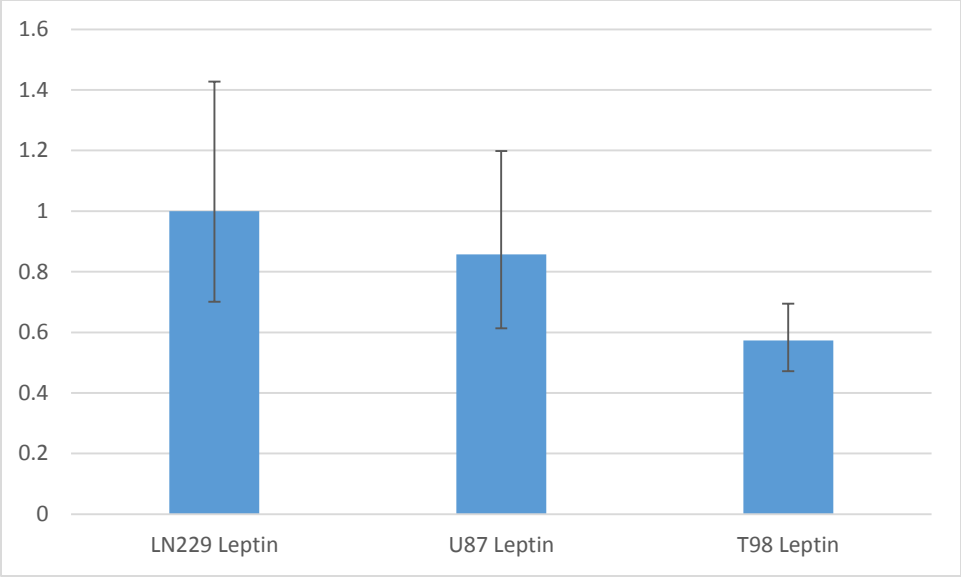

Figure 7: Comparison of leptin mRNA levels in LN229, U87 and T98 GBM cell lines. Leptin normalized relative to beta actin using the $2^{-\Delta\Delta CT}$ method with cell lines leptin levels compared to LN229 leptin.

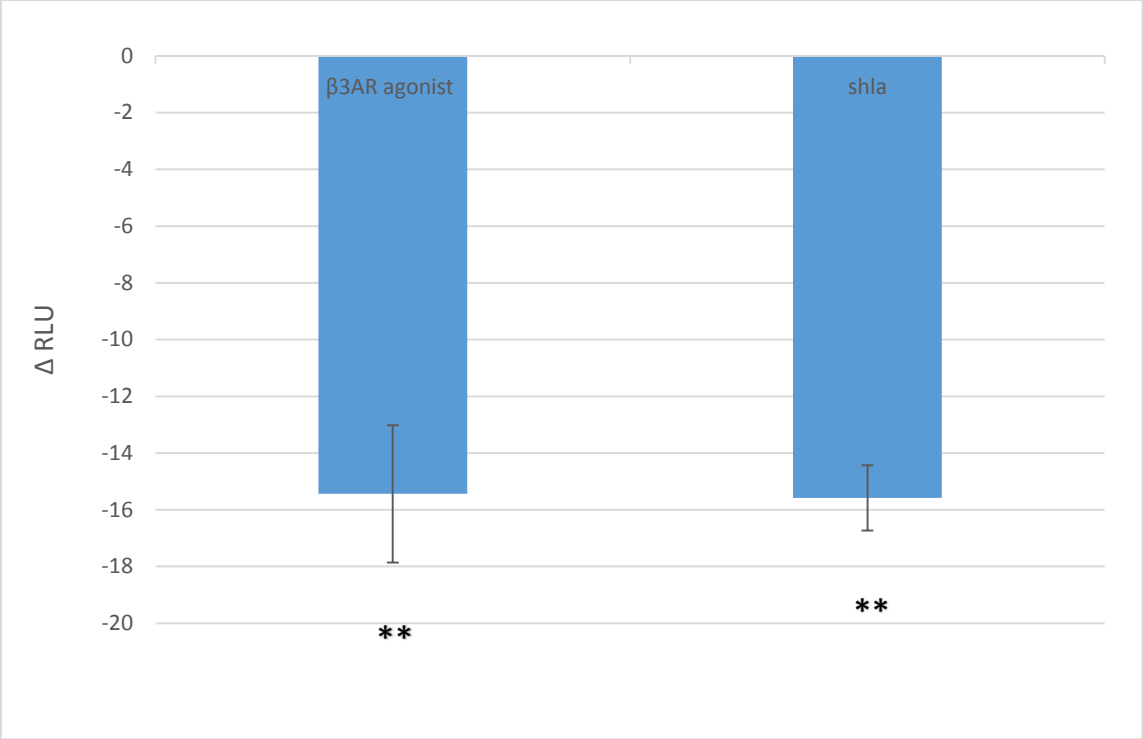

Figure 8: Effects of β3AR agonist and super human leptin antagonist on proliferation of LN229 cell line. LN229 GBM cell line plated at a density of 5,000 cells per well and allowed to adhere over 24 hours. Media was removed and cells were treated with 400 ng/ml of BRL37344 (β-3 adrenergic receptor agonist) and 200 ng/ml super human leptin antagonist. After 48 hours of treatment relative cell number was determined using Cell Titer-Glo by using relative light units using a Modulus Microplate Reader. Δ RLU (the change in relative light units) was measured as compared to control. Analysis includes 4 plates. Significance from control shown by double asterisk (**) represents $p < 0.01$.

CHAPTER FOUR: DISCUSSION

Novel Findings

This investigation started with examining if leptin, leptin receptor and β_3AR were expressed by GBM cell lines. This is the first reported finding of β_3AR mRNA being present in GBM cell lines. While leptin and leptin receptor have previously been reported in rat C6 GBM cells and human GBMs, it is reconfirmed here. Following the finding of β_3AR mRNA expression in GBM cell lines, activation of the β_3AR down regulated leptin expression. This study demonstrated that β3AR was both functional and that activation of the receptor with a specific β3AR agonist resulted in decreased leptin and leptin receptor in LN229 glioma cells. B_3AR agonist treatment decreased cell proliferation as did the SHLA. These results indicated that targeting leptin signaling directly or indirectly may be beneficial in GBM.

Exogenous Leptin

Addition of leptin to GBM cell lines had no overall effect. The lack of effect could be caused by saturation of the leptin receptor by endogenous leptin. It is also possible that the FBS used in culture of the cells may provide an additional source of leptin. It is well established that FBS contains a number of growth factors and other proteins, with its established role during development it would not be surprising if leptin is present in FBS. To observe the effects of exogenous leptin it may be necessary to deplete endogenous leptin (one possible method would be to use a short hairpin RNA for leptin) and assure that unwanted sources of exogenous leptin were eliminated. While exogenous leptin did not promote proliferation endogenous leptin is required for cell survival and continued cell growth.

Adrenergic Stimulation

Nonspecific adrenergic receptor activation in GBM cell lines using epinephrine increased proliferation by 10% at levels consistent with basal (0.025 ng/ml -0.075 ng/ml) and serum stress levels (0.3 ng/ml - 0.5 ng/ml) of epinephrine. While leptin treatment alone produced little to no change in growth, combined treatment of leptin and epinephrine resulted in a significant decrease in proliferation of 6% -7%. If the decrease in proliferation seen with the combined treatment was due to activation of the β_3AR a similar response would be expected with epinephrine alone. To observe the effects of β_3AR in GBM cells a selective agonist, BRL37344, was used to treat the GBM cell lines.

When β_3AR was activated by BRL37344 proliferation decreased between 10% (T98) and 15% (LN229) in following 48 hours of treatment. Treatment with BRL37344 also decreased leptin mRNA by 3%-34% and leptin receptor mRNA by 36%- 52%. The largest decrease in proliferation (15%) occurred in LN229 GBM cells which proportionally have the highest expression of leptin as observed by quantitative PCR. Proliferation of T98 GBM cell line decreased by 10% following treatment with BRL37344 and experienced a 3% change in leptin mRNA and decreased leptin receptor by 52% on average. LN229 GBM cell line decreased proliferation by 15% following treatment with BRL37344 and leptin mRNA levels decreased 34% and leptin receptor mRNA levels decreased 36%.

Examination of relative cell numbers using ATP as an indicator of live cells does not provide insight into the underlying cause of change in cell growth. It is not clear if the changes seen were due to decreased proliferation or to increased apoptosis. Both proliferation and apoptosis have been implicated as an oncomodulatory role of leptin in

other cancer types[37-40]. This study demonstrates that the GBM cell lines examined express leptin and leptin receptor, however the levels of leptin and leptin receptor has been shown to be variable and we suspect that inhibiting cell lines with greater expression of leptin would be the most susceptible to anti-leptin therapy.

Leptin Antagonist

Blocking the leptin receptor using SHLA decreased LN229 cell proliferation by 15% similar to BRL37344. This decrease in proliferation following SHLA treatment provides further evidence of the role of endogenous leptin in GBM cell survival and proliferation. To fully appreciate the effectiveness of SHLA it will be necessary to examine changes in the leptin induced activation of the JAK-STAT pathway. The activation status of the pathway could be examined by monitoring the phosphorylation status of STAT3.

Summary

While exogenous leptin did not increase cell number or proliferation, endogenous leptin appears to be important for continued cell growth. Leptin's oncomodulatory role in GBM cell lines was demonstrated following the administration of BRL37344 or by blocking leptin from binding to leptin receptor. We report that blocking leptin receptor activation and decreasing leptin and leptin receptor levels significantly decreases GBM cell viability. The effectiveness of an anti-leptin therapy appears to be dependent on leptin expression levels. While the proportion of GBM tumors that express leptin and leptin receptor are relatively high (80%) some tumors may not be responsive to an anti-leptin therapy[6]. However, with the evidence provided in this study that leptin plays an

oncomodulatory role in GBM it seems prudent to continue to investigate anti-leptin treatment strategies.

FUTURE DIRECTIONS

β_3AR Agonist

While leptin and leptin receptor mRNA levels decreased with BRL37344 treatment, this may not be a suitable option for GBM patients. One of the many problems in treating GBM is getting drugs to pass through the blood brain barrier and to have high enough concentrations in the region of the tumor yet not be cytotoxic to the rest of the body. While the β_3AR agonist BRL 37344 ability to cross the blood brain barrier is unknown, another selective β_3AR agonist (Amibegron, SR-58611A) is known to readily cross the blood brain barrier and has gone through phase III clinical trials as a treatment for depression[54]. Studies determining the efficacy of Amibegron on tumor cells and alterations in leptin and leptin receptor expression are warranted.

Metformin

A new therapy in GBM is the diabetic drug metformin. In clinical trials metformin has given moderate increases in overall survival 14.6 months vs. 12.1 months and increased 2 year survival by 16% in GBM patients[55]. Metformin decreases serum leptin levels in humans[56] and can reduce leptin levels below resistance in high fat diet (obese) rodents[57]. Also metformin has been shown to decrease growth and migration related to leptin stimulation in GBM cell lines[58]. One final finding of the effects of metformin on GBM is the inhibition of tumor initiating cells or cancer stem cells (CSC)[59]. When treated with metformin CSC showed decreased proliferation and sphere

formation was inhibited[59]. These observed effects may also be due to metformin's effects on leptin. Not only are the effects of down regulating or inhibiting leptin signaling in established GBM cell lines which gives reason to investigate anti-leptin therapy further but emerging data in CSC would point at leptin as an important target in stopping recurrence.

Cancer Stem Cells and Leptin

CSC are a small population of cells that are resistant to current therapies that are the cause of recurrence in GBM. CSCs are able to recapitulate the tumor when a small number of cells (< 100) were injected into mice, while differentiated tumor cells are unable to form a tumor[60]. A recent study has shown CSC overexpress leptin receptor to a greater degree in GBM compared to differentiated tumor cells[61]. Breast CSC also have higher levels of leptin receptor compared to the differentiated tumor cells[62]. In mammary tumors, leptin is needed for stem cell proliferation and renewal[62]. Breast cancer cells derived from transgenic mice and then injected orthotopically into leptin deficient mice (ob/ob mice) did not form tumors as effectively as wild type mice[63]. Also db/db mice (obese mice with high leptin but nonfunctional leptin receptors) showed increased tumorigenesis when given orthotopical injections of cancer stem cells compared to wild type obese mice. A related finding has been observed in GBM CSC with inhibition of Stat3 inhibiting proliferation and CSC could not recover the ability to self-renew after Stat3 inhibition[64]. Due to these findings leptin will continue to be an important area of research for the treatment of aggressive recurring cancers like GBM.

Conclusion

In conclusion we have demonstrated that inhibiting leptin has anti-proliferative effects, which in itself may be a reason to further investigate leptin as an adjuvant therapy for GBM. While current experimental drugs for GBM, like metformin, may actually be targeting leptin further work investigating metformin's effects on leptin in GBM are warranted. This study also demonstrates that treating GBM with a β_3AR agonist may be equally valid based upon the number of GBMs expressing β_3AR. New findings of leptin's role in CSC proliferation and self-renewal makes leptin and even more promising target as CSC are resistant to current treatments for GBM.

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Formatting of citations in accordance with the journal Cell.

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APPENDIX A (STATISTICAL ANALYSIS OUTPUT)

SPSS

STATISTICS APPENDIX

Descriptives

RLU change from untreated

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	84	-.0400	.12055	.01315	-.0662	-.0138	-.39	.30
2.00	84	-.0953	.10390	.01134	-.1179	-.0728	-.40	.10
3.00	84	-.0996	.08316	.00907	-.1177	-.0816	-.39	.11
Total	252	-.0783	.10678	.00673	-.0916	-.0651	-.40	.30

Table 2: Statistical descriptive of T98 GBM cell line treated with 1. leptin (200 ng/ml), 2. leptin (200 ng/ml) in combination with β_3AR agonist (400 ng/ml), 3. β_3AR agonist (400 ng/ml).

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.186	2	.093	8.643	.000
Within Groups	2.676	249	.011		
Total	2.862	251			

Table 3: One way ANOVA comparing T98 GBM cell line change in RLU between treatments (leptin, leptin and β_3AR agonist, and β_3AR agonist).

Multiple Comparisons

Dependent Variable: RLU change from untreated

	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	1.00	2.00	.05534*	.01600	.002	.0176	.0931
		3.00	.05962*	.01600	.001	.0219	.0973
	2.00	1.00	-.05534*	.01600	.002	-.0931	-.0176
		3.00	.00427	.01600	.961	-.0334	.0420
	3.00	1.00	-.05962*	.01600	.001	-.0973	-.0219
		2.00	-.00427	.01600	.961	-.0420	.0334

Table 4: Tukey HSD post hoc analysis of T98 GBM cell line treatment RLU change from untreated with the following treatments: treated with 1. leptin (200 ng/ml), 2. leptin (200 ng/ml) in combination with β_3AR agonist (400 ng/ml), 3. β_3AR agonist (400 ng/ml).

Descriptives

RLU change from untreated

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	60	.0181	.12332	.01592	-.0137	.0500	-.39	.21
2.00	36	-.0292	.18485	.03081	-.0917	.0334	-.50	.48
3.00	42	.0966	.08220	.01268	.0710	.1223	-.06	.26
4.00	42	.1023	.09542	.01472	.0726	.1320	-.18	.30
5.00	36	-.0689	.16162	.02694	-.1236	-.0142	-.43	.16
6.00	35	-.0734	.12647	.02138	-.1168	-.0299	-.46	.11
Total	251	.0133	.14695	.00928	-.0049	.0316	-.50	.48

Table 5: Statistical descriptive of T98 treated with 1. leptin treatment (200 ng/ml), 2. leptin treatment (400 ng/ml), 3. epinephrine at basal levels, (0.025 ng/ml – 0.075 ng/ml), 4. epinephrine at high stress levels (0.3 ng/ml -0.5 ng/ml), 5. leptin (200 ng/ml) in

combination with basal levels of epinephrine, 6. leptin (200 ng/ml) in combination with high stress levels of epinephrine.

ANOVA

deltaRLU

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.197	5	.239	13.959	.000
Within Groups	4.201	245	.017		
Total	5.398	250			

Table 6: One way Anova of change in RLU in T98 GBM cell line between treatments leptin, epinephrine at basal levels, epinephrine at high stress levels, leptin and basal levels of epinephrine, leptin and high stress levels of epinephrine.

Multiple Comparisons

Dependent Variable: deltaRLU

Tukey HSD

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.04730	.02761	.524	-.0320	.1266
	3.00	-.07850*	.02635	.037	-.1542	-.0028
	4.00	-.08417*	.02635	.020	-.1598	-.0085
	5.00	.08702*	.02761	.022	.0077	.1663
	6.00	.09153*	.02785	.015	.0115	.1715
2.00	1.00	-.04730	.02761	.524	-.1266	.0320
	3.00	-.12580*	.02974	.000	-.2112	-.0404
	4.00	-.13146*	.02974	.000	-.2169	-.0460
	5.00	.03972	.03087	.792	-.0489	.1284
	6.00	.04423	.03109	.713	-.0451	.1335
3.00	1.00	.07850*	.02635	.037	.0028	.1542
	2.00	.12580*	.02974	.000	.0404	.2112
	4.00	-.00567	.02858	1.000	-.0878	.0764
	5.00	.16552*	.02974	.000	.0801	.2510
	6.00	.17002*	.02997	.000	.0839	.2561
4.00	1.00	.08417*	.02635	.020	.0085	.1598
	2.00	.13146*	.02974	.000	.0460	.2169
	3.00	.00567	.02858	1.000	-.0764	.0878
	5.00	.17119*	.02974	.000	.0858	.2566

	6.00	.17569*	.02997	.000	.0896	.2618
	1.00	-.08702*	.02761	.022	-.1663	-.0077
	2.00	-.03972	.03087	.792	-.1284	.0489
5.00	3.00	-.16552*	.02974	.000	-.2510	-.0801
	4.00	-.17119*	.02974	.000	-.2566	-.0858
	6.00	.00451	.03109	1.000	-.0848	.0938
	1.00	-.09153*	.02785	.015	-.1715	-.0115
	2.00	-.04423	.03109	.713	-.1335	.0451
6.00	3.00	-.17002*	.02997	.000	-.2561	-.0839
	4.00	-.17569*	.02997	.000	-.2618	-.0896
	5.00	-.00451	.03109	1.000	-.0938	.0848

Table 7: Tukey HSD post hoc analysis of T98 GBM cell line treatment RLU change from untreated with the following treatments: 1. leptin treatment (200 ng/ml), 2. leptin treatment (400 ng/ml), 3. epinephrine at basal levels, (0.025 ng/ml – 0.075 ng/ml), 4. epinephrine at high stress levels (0.3 ng/ml -0.5 ng/ml), 5. leptin (200 ng/ml) in combination with basal levels of epinephrine, 6. leptin (200 ng/ml) in combination with high stress levels of epinephrine.

SHLA and b3 ln229 against control (0) 2 tailed

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
SHLA	30	-.1558	.06292	.01149
β_3AR	24	-.1544	.11866	.02422

Table 8: Statistical description of SHLA and β_3AR agonist treated LN229 against control.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
SHLA	-13.562	29	.000	-.15580	-.1793	-.1323
β_3AR	-6.374	23	.000	-.15438	-.2045	-.1043

Table 9: One sample t-test comparing SHLA and β_3AR agonist treated LN229 against control.

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
LN229 β_3AR	24	-.1544	.11866	.02422
T98 β_3AR	84	-.0996	.08316	.00907

Table 10: Statistical description of β_3AR agonist treated LN229 and T98 against control.

One-Sample Test

	Test Value = 0					
	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
LN229 β_3AR	-6.374	23	.000	-.15438	-.2045	-.1043
T98 β_3AR	-10.979	83	.000	-.09961	-.1177	-.0816

Table 11: One sample t-test comparing β_3AR agonist treated LN229 and T98 to control.