GLIOMA STEM-LIKE CELL SURVIVAL IS AFFECTED BY THEIR MACROPINOCYTIC UPTAKE AND TARGETED TRAFFICKING OF BEVACIZUMAB

A dissertation submitted
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by

Gaëlle Melanie Müller-Greven

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Dissertation written by
Gaëlle Melanie Müller-Greven
B.S., McGill University, 2010
Ph.D., Kent State University, 2018

Approved by

Candece L. Gladson, M.D. __________, Chair, Doctoral Dissertation Committee
Gail C. Fraizer, Ph.D. __________, Members, Doctoral Dissertation Committee
Edgar E. Kooijman, Ph.D. __________,
Robert H. Silverman, Ph.D. __________,
Douglas Kline, Ph.D. __________,

Accepted by

Ernest J. Freeman, Ph.D. __________, Chair, School of Biomedical Sciences
James L. Blank, Ph.D. __________, Dean, College of Arts and Sciences
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CHAPTER

1

INTRODUCTION

Malignant Glioma

Glioblastoma multiform (GBM), a grade IV astrocytoma, is the most common and lethal cancer of the central nervous system. In the United States, approximately 21 out of 100,000 individuals will be diagnosed with a primary brain tumor, with seven of them being gliomas and half of those being glioblastomas. (Weller et al., 2015) Animal studies suggest that glioblastomas largely arise from precursor cells in the brain, or a stem-like cell, but they can also arise from a differentiated astrocyte. (Alcantara Llaguno et al., 2009, 2015; Chen et al., 2012; Dai et al., 2001; Lindberg et al., 2009)
No risk factors have been elucidated for the majority of glioblastomas diagnosed, except for a small percentage of patients having a family history of brain tumors or from exposure to medically relevant levels of ionizing radiation. Studies have shown a potential relationship between increased levels of IgE and a lower risk of gliomas. Glioblastoma is most prevalent in older patients with an average age at onset of 64 years. (Weller et al., 2015)

There has been limited improvement in life expectancy for patients with glioblastoma, with almost all tumors recurring and contributing to the overall survival of approximately 15 months. Currently, most patients with primary glioblastoma have resection when possible followed by radiation and chemotherapy with temozolomide. One of the difficulties in determining the efficacy of new therapeutic agents is that many clinical trials do not include control groups and instead rely on comparisons to historical data. (Gorlia et al., 2012)

A review of eight phase I/II clinical trials carried out between 1999 and 2010 by the European Organization for Research and Treatment of Cancer (EORTC) Brain Tumor Group (BTG) for recurrent glioblastoma treatments that were ultimately deemed to have no clinical activity provides pooled progression-free survival (PFS) and overall survival (OS) data that can be used for baseline comparison against new therapeutic agents. Based on the pooled data, the overall median PFS for recurrent glioblastoma was found to be 1.8 months (1.7 months - 1.9 months), the percent of patients with PFS at six months (PFS-6) was 14.7% (11.0% - 19.0%), the median OS was 6.2 months (5.7 months - 7.1 months), and the percent of patients surviving at 12 months (OS-12) was 22.1% (17.5% - 27.0%). (Gorlia et al., 2012)
The majority of glioblastoma studies focus on primary tumors and thus much less is known about recurrent tumors, which are more difficult to study in part due to limited access to tissue samples. There is currently no standard of care for recurrent (also known as progressive) glioblastoma, and oftentimes treatments given are based on their efficacy in primary tumors, which behave much differently. (Campos et al., 2016)

Glioblastoma tumors can be categorized by several common genetic characteristics including (epidermal growth factor receptor) EGFR amplification, (phosphatase and tensin homolog) PTEN mutation or loss, (epidermal growth factor receptor variant III) EGFRvIII expression, loss of the chromosome 10q arm, and gain of the chromosome 7q arm. (Weller et al., 2015) However, glioblastomas are very heterogeneous; the complex genetic diversity is thought to prevent current therapies from eliminating the entire tumor and increases the likelihood for recurrence and resistance.

Selection pressure from therapies and the microenvironment may actually play a role in promoting heterogeneous populations of tumor cells within the same tumor as well as within local and distant recurrent tumors. (Campos et al., 2016) Distant recurrences have much greater genomic differences to their paired primary tumors at the time of surgery, suggesting that glioma tumor cells disperse early and likely are located outside of the resected sections. (Kim et al., 2015a, 2015b) In addition, therapies such as temozolomide have been shown to drive tumor evolution. (Johnson et al., 2014) Single-cell RNA-sequencing of 430 individual cells from five primary glioblastoma tumors provided a high-resolution view of the large extent of intra-tumoral heterogeneity. (Patel et al., 2014)
Glioblastoma tumors are characterized by their high vascularity with high microvessel density and dis-organized blood vessels. (Brem et al., 1972) Angiogenesis is a logical pathway to target for the treatment of glioblastoma, and several therapeutics that inhibit angiogenesis have been developed including bevacizumab. Currently, no anti-angiogenic therapy has demonstrated meaningful improvement in patient OS, however, when patients are selected based on specific imaging characteristics, treatment with bevacizumab increases PFS and OS. Humanized IgG (hIgG) monoclonal antibodies such as bevacizumab are now used in cancer therapy, although surprisingly little is known concerning their access to the perivascular space in the brain and the mechanisms by which they affect tumor cells within this microenvironment.

Glioblastoma Standard of Care and Bevacizumab

The current standard of care for treating patients with primary glioblastoma includes daily irradiation (2 Gy/fraction) Monday through Friday with daily concomitant treatment of temozolomide (75 mg/m²/day administered orally) seven days per week for a total of six weeks and 60 Gy, followed by adjuvant treatment consisting of six 28-day cycles of temozolomide at 150mg/m²/day administered orally for five consecutive days in the first cycle and increased to 200mg/m²/day for five consecutive days for the remaining cycles. (Stupp et al., 2005) The median overall survival increased in glioblastoma patient populations between 2000-2003 and 2005-2008 from 8.1 months to 9.7 months. This increase in median OS is likely due to the implementation of the standard of care. The standard of care was introduced after a phase III
clinical trial demonstrated that the combination of temozolomide with radiation therapy increased survival of newly-diagnosed glioblastoma to 14.6 months from 12.1 months when radiation was used alone. (Johnson and O’Neill, 2012; Stupp et al., 2005; Weller et al., 2013)

For recurrent glioblastoma, temozolomide was FDA approved in 1999 based on two phase II clinical trials that showed increased PFS with temozolomide. (Johnson and O’Neill, 2012; Seystahl et al., 2016) The first study concluded that temozolomide treatment (150-200 mg/m²/day administered orally for 5 consecutive days, repeated every 28 days) was superior to procarbazine treatment (125-150mg/m²/day administered orally for 28 consecutive days, repeated every 56 days) in recurrent glioblastoma patients enrolled between 1995 and 1997 based on increased PFS at 6 months (PFS-6) of 21% and 8%, respectively. The study also determined that temozolomide had a slight increase in OS of 1.5 months but that the results were not statistically significant. (Yung et al., 2000) The second study was a one-arm clinical trial with recurrent glioblastoma patients enrolled between 1995 and 1996 that concluded temozolomide treatment, following a regimen similar to that of the Yung et. al study, moderately improved PFS-6 (19%) based on a comparison to historical data. (Brada et al., 2001)

Temozolomide is a second generation imidazotetrazine derivative that converts to 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MITC), a cytotoxic DNA alkylating agent that adds methyl groups to purine bases of DNA causing DNA damage-induced apoptosis. (Zhang et al., 2012) Temozolomide does not require hepatic activation as does procarbazine, a chemotherapeutic agent that was commonly administered for treatment of recurrent glioblastoma before phase II studies demonstrated superior PFS and reduced cytotoxicity by treatment with temozolomide.
(Brada et al., 2001) Glioblastoma patients who have a methylated promoter for the DNA repair protein O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) respond much better to alkylating agents such as temozolomide, as MGMT is not expressed and therefore cannot reverse methyl groups added by the alkylating agents, ultimately leading to greater tumor cell death compared to patients with an unmethylated MGMT promoter. (Esteller et al., 2000; Hegi et al., 2004, 2005)

Due to the characteristically high vascularity of glioblastoma tumors, angiogenesis was a promising target to eradicate tumors. The concept of inhibiting angiogenesis for the treatment of cancer first appeared over 40 years ago, however at the time there was little information on which molecules were pro-angiogenic. Several important pro-angiogenic factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) were discovered but a major breakthrough occurred when vascular endothelial growth factor (VEGF, also VEGF-A) was discovered by Ferrara and colleagues as well as Connolly and colleagues in 1989. VEGF is a key regulator of angiogenesis because it is a rate-limiting step, and is itself regulated by the microenvironment. Alternative splicing creates several isoforms, however isoform VEGF\textsubscript{165} appears to be the most physiologically relevant for angiogenesis. There are two receptors that bind to VEGF, vascular endothelial growth factor receptor 1 (VEGFR1 or Flt-1) and VEGFR2 (Flk-1 or KDR), of which VEGFR2 has the greatest influence on angiogenesis. VEGF is over-expressed in many different cancers including glioblastoma (Berkman et al., 1993; Yao et al., 2001) and its inhibition temporarily normalizes the vasculature. The term vascular “normalization” observed with inhibitors of VEGF has been used to describe blood vessel pruning and decrease vessel tortuosity, with resultant increased oxygenation, chemotherapy delivery, interstitial pressure, and permeability to macromolecules. (Ferrara et al., 2004)
VEGF was thought early on to act solely on vascular endothelial cells, and thus it was thought that blocking VEGF would prevent tumor growth through a lack of new blood vessels that are required to meet the oxygen and nutrient demands of a growing tumor. (Ferrara and Henzel, 2012; Kim et al., 1993)

Bevacizumab (Avastin®) is a recombinant humanized monoclonal IgG antibody that was developed by Ferrara and colleagues at Genentech in 1997. Bevacizumab binds to VEGF with high affinity ($K_d \sim 0.5\text{nM}$) at Gly88, a human-specific residue, and effectively neutralizes all isoforms of VEGF-A. Advantages of a monoclonal anti-angiogenic agent include extended half-life (17-21 days) and binding stability as compared to other therapeutic methods such as small molecule inhibitors, dominant-negative mutants, and anti-sense oligos to the VEGF gene. (Ferrara et al., 2004)

Following safety testing in animals, Phase I clinical trials with bevacizumab began in 1997 and concluded that the monoclonal antibody (mAb) was not toxic to humans. Shortly after, Phase II clinical trials were carried out in patients with prostate cancer, renal-cell cancer, non-small-cell lung cancer, and metastatic breast and colon cancer. (Ferrara et al., 2004) By 2004, bevacizumab combination therapy was approved by the United States Food and Drug Administration (FDA) for metastatic colon and rectal cancer. (Braghiroli et al., 2012)

Bevacizumab received accelerated FDA approved as a treatment for recurrent glioblastoma in 2009 based on increased six-month PFS found in two Phase II clinical trials that did not have
control groups. Additional clinical trials have been completed showing that although there is a slight increase in median PFS in non-selected patients, bevacizumab did not improve median OS. (Seystahl et al., 2016) Patients with recurrent glioblastoma are now routinely treated with bevacizumab as it has some clinical benefit for patients such as inducing normalization of the tumor vasculature, reduces edema, and enhances quality-of-life. (Lu-Emerson et al., 2015)

In an effort to improve OS of patients with recurrent glioblastoma treated with bevacizumab, salvage and combination treatments have been evaluated in clinical trial studies including lomustine, onartuzumab, rilotumumab, trebananib, vorinostat, and sunitinib, but these studies did not select patients with specific imaging characteristics.

A retrospective study evaluated whether bevacizumab in combination with lomustine, an alkylating agent similar to temozolomide, improved PFS and OS in recurrent glioblastoma patients compared to bevacizumab as a monotherapy. The combination therapy significantly increased median PFS (6.1 months vs. 2.3 months), PFS-6 (50% vs. 12%), and median OS (6.6 months vs. 4.1 months) compared to bevacizumab monotherapy. (Heiland et al., 2016)

Recently, bevacizumab received full FDA approval for treatment of recurrent glioblastoma following the results of a randomized phase III clinical trial that followed 437 patients at first recurrence from 2001 through 2014 at 38 institutions globally who were treated with either lomustine alone or a combination of bevacizumab and lomustine. The study found that, compared to lomustine alone, the combination of bevacizumab and lomustine significantly
increased median PFS (4.2 months vs. 1.5 months), although there was no difference in OS (9.1 months vs. 8.6 months). (Wick et al., 2017)

Resistance to bevacizumab treatment could occur through a switch to VEGF-independent pro-angiogenic signaling pathways where receptor kinases other than VEGFR are activated such as c-Met, the receptor for hepatocyte growth factor (HGF). In an effort to increase the efficacy of bevacizumab for the treatment of recurrent glioblastoma, a multi-center randomized clinical study that evaluated the combination of bevacizumab plus onartuzumab, a monoclonal antibody that blocks the MET receptor, and bevacizumab plus placebo was performed. The combination of bevacizumab plus onartuzumab did not overall improve patient outcomes as compared to bevacizumab plus placebo based on median PFS and median OS. (Cloughesy et al., 2017)

Another monoclonal antibody, rilotumumab, was developed to block HGF, the ligand for the MET receptor. One phase II study evaluated rilotumumab in approximately 60 relapsed glioblastoma patients, half of whom had previously been treated with bevacizumab. The median PFS was slightly more than four weeks for both bevacizumab-naïve patients and those previously treated with bevacizumab, however bevacizumab-naïve patients had a greater median OS (approximately 11 months vs. 3.5 months), suggesting that resistance to bevacizumab may have impacted patient response to rilotumumab. (Wen et al., 2011) Another phase II study evaluated the combination of bevacizumab and rilotumumab in a one-arm study design with 36 recurrent glioblastoma patients enrolled. The median OS was 11.2 months, similar to bevacizumab plus placebo in the clinical trial evaluating the combination of bevacizumab plus
onartuzumab, however there was a slight improvement in PFS-6 compared to other combination therapies evaluated. (Peters and Affronti)

In addition to VEGF signaling, angiopoietin (Ang) 1 and 2 regulate angiogenesis through their tyrosine kinase with immunoglobulin-like and EGF-like domains (Tie) receptors that are largely expressed exclusively on endothelial cells. The increased expression of Ang1, Ang2, and Tie2 in high-grade astrocytoma suggests that this signaling pathway may be involved in the resistance to bevacizumab treatment. (Audero et al., 2001; Hatva et al., 1995; Scholz et al., 2016) In an attempt to reduce recurrent glioblastoma resistance to bevacizumab treatment, a phase II clinical study was conducted to determine if a combination of bevacizumab and trebananib (AMG 386), an Ang1/2 neutralizing peptide, would improve patient outcomes. The combination of bevacizumab plus AMG 386 did not improve patient outcomes as compared to bevacizumab plus placebo based on median PFS, PFS-6, and median OS. (Lee)

Other mechanisms to inhibit angiogenesis have been identified, including the anti-angiogenic actions of histone deacetylase (HDAC) inhibitors based on the down-regulation of pro-angiogenic genes known to be regulated by HDACs. (Mottet and Castronovo, 2010) A phase II, non-randomized single-arm study evaluated 40 recurrent glioblastoma patients with a combination of bevacizumab and vorinostat, a small molecule HDAC inhibitor. (Grant et al., 2007) Compared to historical data, the combination did not improve patient outcomes based on median PFS, and median OS. (Ghiaseddin et al., 2017)
In other efforts to improve recurrent glioblastoma patient outcomes, broadly-acting small molecule inhibitors such as sunitinib have also been studied in the clinic. Sunitinib inhibits several receptor tyrosine kinases including platelet-derived growth factor receptors (PDGFR) alpha and beta, and VEGFR1, VEGFR2, and VEGFR3. (Mendel et al., 2003) A phase II clinical trial evaluated whether sunitinib could improve patient outcomes for patients previously treated with bevacizumab or not. Bevacizumab-naïve patients had a slightly longer time to tumor progression than did the bevacizumab resistant patients and improved overall survival. (Kreisl et al., 2013) The greater time to progression and longer overall survival in bevacizumab-naïve recurrent glioblastoma patients in this study as well as in the rilotumumab study are evidence of the complexities regarding bevacizumab-induced resistance. Bevacizumab-resistant tumors are more aggressive and difficult to treat with current therapies, and they remain a challenge for clinicians and researchers.

Several ongoing and recent clinical studies have suggested promising new approaches to improve patient outcomes. One ongoing clinical study involves the combination of bevacizumab and VB-111 (GLOBE study NCT02511405). VB-111 is a non-replicating adenovirus with a chimeric protein comprised of Fas and tumor necrosis factor receptor 1 (TNFR1) with a modified murine pre-proendothelin promoter that will only be transcribed in developing endothelial cells. The chimera will induce apoptosis of activated endothelial cells upon endogenous circulating TNFα binding thus inhibiting angiogenesis. One benefit of this therapy is the lack of a need to cross the blood-brain barrier since the adenovirus only needs to enter the endothelial cells. (Gruslova et al., 2015) Another promising study that was recently completed is the ReACT study (NCT01498328) that evaluated the combination of bevacizumab and rindopepimut, a
KLH-conjugated peptide that binds to mutated EGFRvIII, a protein almost exclusively expressed in glioblastoma tumor cells in a subgroup of patients, to elicit an immune response specifically against those tumor cells. (Gedeon et al., 2013)

There are limited reports of studies evaluating the direct effect of bevacizumab on tumor cells. One group found that bevacizumab slightly enhanced the proliferation of several glioblastoma cell lines, similar to the effect observed with VEGF-A. Interestingly, bevacizumab alone slightly increased the phosphorylation of Protein Kinase B (PKB, also known as Akt) and extracellular signal–regulated kinases (ERK), kinases that are downstream from VEGFR1 and VEGFR2, possibly through a compensatory pathway. Selective Akt and ERK inhibitors diminished the slightly enhanced proliferation caused by bevacizumab, suggesting that the proliferative effects of bevacizumab partly depend on those pathways. (Simon et al., 2014)

As eluded to previously, two modes of resistance to anti-angiogenic therapy are thought to exist: extrinsic or evasive resistance, caused by selection pressures from treatments and the microenvironment, and intrinsic resistance or indifference to anti-angiogenic therapy caused by altered pathways in the tumor. (Bergers and Hanahan, 2008) The therapy selected for the treatment of glioblastoma tumors may also depend on the tumor molecular subtype, as clinical outcomes have been shown to vary by molecular subtype. Based on the genomic analysis of 200 glioblastoma samples cataloged by The Cancer Genome Atlas (TCGA), tumors were clustered into the classical, mesenchymal, and neural subtypes. (Verhaak et al., 2010)
Extrinsic resistance can occur through several different mechanisms including increased expression of pro-angiogenic factors such as fibroblast growth factor 2 (FGF2) and angiopoietin 2 (Ang2) to promote angiogenesis through VEGF-independent routes (Casanovas et al., 2005; Labussière et al., 2016), recruitment of bone marrow-derived progenitor cells due to increases in hypoxia-inducible factor 1 alpha (HIF-1a) and stromal-derived factor-1 (SDF1) (Du et al., 2008; Piao et al., 2012; Shojai et al., 2007), increased pericyte recruitment and coverage to support endothelial cells (Darland et al., 2003; Franco et al., 2011; Kumar et al., 2013; Mancuso et al., 2006; Thomas et al., 2013), and increased tumor invasion and metastasis through vascular co-option. (Du et al., 2008; Narayana et al., 2009; Rubenstein et al., 2000)

Therapy-driven evolution, as has been shown in temozolomide-treated glioblastomas that have been shown to bear higher mutation rates than temozolomide-naive tumors, increases resistance to, and create a challenge for, targeted therapies. Analysis of somatic mutations following temozolomide treatment in recurrent gliomas reveal nucleotide base pair transitions that are characteristic of alkylating agents, namely G>A and C>T, that can confer tumor evasion to therapy especially when genes important for DNA mismatch repair are mutated, leading to hypermutations without apoptosis. (Hunter et al., 2006; Johnson et al., 2014)

Intrinsic resistance, or simply indifference to anti-angiogenic therapies, is much less studied especially in the context of glioblastoma. Several mechanisms of intrinsic resistance have been proposed including selection pressure from the microenvironment such that tumor progression occurs without the need for VEGF signaling before the introduction of anti-VEGF drugs, which at that point would provide no clinical benefit. In addition, the microenvironment could be
primed with bone marrow-derived myeloid cells that were recruited prior to anti-angiogenic therapy. Other proposed mechanisms involve patient-specific genetic alterations in genes related to the pathways affected by anti-angiogenic therapies. (Bergers and Hanahan, 2008) Cancer stem-like cells have also been implicated in resistance to both radiation therapy and chemotherapy. (Bao et al., 2006a; Chen et al., 2012)

In common with other humanized monoclonal antibodies, the response to bevacizumab therapy shows inter-individual variability and approximately 20% of patients with recurrent glioblastoma do not show any response. (Nowosielski et al., 2014) The reasons for this lack of response have not been elucidated and the effects of bevacizumab on the tumor cells in the perivascular niche have not been determined.

The Blood-Brain Barrier and Challenges with Therapeutic Delivery

The vascular system is critical for the development and maintenance of normal tissues to supply oxygen and nutrients to cells and to remove metabolic waste products. For the same reasons, tumors require a functional vascular system to supply their high demands for oxygen and nutrients while removing waste from the growing new mass of cells. However, the vasculature in the normal and tumor brain differ in multiple ways that affect the treatment response for glioblastoma patients. (Dudley, 2012)
Normal blood vessels have a hierarchal structure; the larger arteries split off into smaller arterioles that then lead to thin capillaries, which supply oxygen and nutrients to all the cells in a healthy tissue. The larger blood vessels are supported by smooth muscle cells while pericytes support the delicate capillaries. (Hirschi and D’Amore, 1996) Pericytes provide support physically as well as through molecular signaling from direct contact with endothelial cells in a paracrine fashion. (Hirschi and D’Amore, 1997) Pericytes rely on the signaling events from the binding of platelet derived growth factor (PDGF) to the PDGFR-α receptor expressed on pericytes for survival and to prevent blood vessel leakage. (Hellström et al., 2001) Pericytes regulate endothelial cell proliferation, as the absence of pericytes in PDGF-B deficient mice resulted in endothelial cell hyperplasia and abnormal blood-brain barrier function based on abnormal cell shape, tight junction protein expression, and increased permeability. (Hellström et al., 2001)

Astrocytes play an important role in supporting endothelial cells and promoting the blood-brain barrier function. Non-neural endothelial cells co-cultured with astrocytes gained blood-brain barrier properties. (Janzer and Raff, 1987) Interestingly, steroids, which are used to reduce edema in glioma patients, have a similar effect of promoting blood-brain barrier properties in endothelial cells as do astrocytes. (Underwood et al., 1999)

The basement membrane of blood vessels provides physical support as well as support through molecular signaling, especially through the integrin receptors on the surfaces of endothelial cells, cancer stem-like cells, and other cells that are located in the perivascular space. The
extracellular matrix in the basement membrane is protein-rich and composed of collagen, laminin, fibronectin, and other proteins as well as proteoglycans and glycosaminoglycans.

New blood vessels can form through several different mechanisms, some of which are typically limited to embryonic development such as vasculogenesis. Two mechanisms of new blood vessel formation that occur in adult tissue and can occur in tumor tissue are angiogenesis and intussusceptive angiogenesis. (Dudley, 2012) Angiogenesis provides a means of reaching new areas of tissue that are not well vascularized and potentially hypoxic, however this method of new blood vessel formation relies on endothelial cell proliferation and is therefore a relatively slow process. Intussusceptive angiogenesis is a rapid process as there is no immediate requirement for endothelial cell proliferation but instead occurs through remodeling of the current vasculature by trans-vascular tissue pillars derived from the originating capillary walls that split the lumen and bifurcate the vessel. (Burri et al., 2004; Dudley, 2012)

Angiogenesis involves the dissolution of the basement membrane followed by endothelial cell proliferation and sprouting of the immature tip cells towards angiogenic growth factors such as VEGF. In normal tissue, there is a gradient of VEGF that promotes a patterned formation of the vascular structure and is self-regulating so that blood vessel networks are fine-tuned to supply nutrients and oxygen evenly. (Dudley, 2012; Gerhardt et al., 2003; Hellström et al., 2001) Glioblastoma tumors chronically express high levels of VEGF that improperly guides new endothelial tip cells due to the lack of a VEGF gradient and results in an disorganized vascular network. (Berkman et al., 1993; Dudley, 2012; Yao et al., 2001)
The tumor vasculature lacks the hierarchal vessel structure seen in normal tissue and is not well supported by smooth muscle cells and pericytes. Pericytes are less abundant and more weakly attached to endothelial cells in glioblastoma tumors. (Abramsson et al., 2002; Baluk et al., 2005) Hypoxic regions are common in glioblastoma tumors as some blood vessels are not stable enough to support proper blood flow, or can exhibit a reverse flow or worse, not be perfused at all. (Kamoun et al., 2010) Angiogenesis may outpace tumor growth initially, but as the process is somewhat slow, the tumor will likely outgrow its vasculature further causing hypoxic regions. (Dudley 2012)

Normal brain endothelial cells form an almost impenetrable barrier between the blood and the brain parenchyma to protect the brain from toxins and pathogens. The main characteristics that distinguish brain microvascular endothelial cells from endothelial cells in other organs, which are more permeable, include the intercellular restriction of molecules by the prevalence of tight junctions and the intracellular restriction of molecules by tightly-regulated endocytosis. The tumor vasculature is leaky due to a lack of proper endothelial cell barrier function. (Daneman and Prat, 2015)

Tumor brain endothelial cells have an irregular shape with long cytoplasmic projections that can sprout into the vessel and create small intercellular gaps. (Dudley, 2012) In addition, tumor brain endothelial cells lack proper formation of intercellular tight junctions due to chronic endothelial cell proliferation, which produces a mosaic pattern of CD31 endothelial cell marker staining indicating sections of vasculature that do not contain endothelial cells. (di Tomaso et al., 2005) Tight junctions are an important feature of the blood-brain barrier. The main
components of tight junctions are the transmembrane proteins occludin and claudin-1/-3/-5/-12, which bind to their counterparts between endothelial cells, and the intracellular proteins zonula occludens (ZO) -1, -2, and -3 that bind to actin. Occluden was the first tight junction protein discovered and appears to mainly play a regulatory role due to its involvement in trans-endothelial migration of neutrophils. (Huber et al., 2000; Wolburg and Lippoldt, 2002) Claudins appear to mainly play a role in barrier function. (Wolburg and Lippoldt, 2002) Tight junctions are often immature or lacking between endothelial cells in brain tumors and this exacerbates the breakdown of the blood-brain barrier. (Dudley, 2012; Liebner et al., 2000; Wolburg et al., 2003)

A major challenge for treating brain tumors is drug delivery to the brain. Although tumor vessels are leaky, many pharmacological drugs have limited penetration to the tumor area. Brain delivery systems are under active investigation. For example, temozolomide placed within a cationic liposome nano-complex conjugated to an anti-transferrin receptor antibody has improved blood-brain barrier penetrance and results in an enhanced anti-tumor effect of temozolomide on cancer cells that over-express the transferrin receptor and decreases cytotoxicity in a mouse model of glioblastoma. (Kim et al., 2015c) The transferrin receptor can be fused with a therapeutic antibody to greatly increase blood-brain barrier penetrance of these large IgG proteins by hijacking the transcytosis pathway of transferrin. (Pardridge, 2015) Alternatively, conjugation of a liposome with the angiopep-2 sequence promotes transcytosis across endothelial cells via the low density lipoprotein receptor-related protein 1 (LRP-1) pathway. (Lu et al., 2017; Tian et al., 2015) Brute-force opening of the blood-brain barrier with mannitol has been shown to reduce the frequency of bevacizumab doses required for the same
anti-tumor effect as the standard bevacizumab treatment regimen in recurrent glioblastoma patients. (Chakraborty et al., 2016)

The Perivascular Space and Cancer Stem-Like Cells

The microenvironment provided in the perivascular space is emerging as a critical determinant of tumor cell growth and survival (reviewed in (Gilbertson and Rich, 2007)). Cells of the perivascular niche include endothelial cells, pericytes, smooth muscle cells, astrocytes, microglia, macrophage, and glioma cancer stem-like cells (CSLCs). As described previously, endothelial cells form blood vessels and are supported by smooth muscle cells and pericytes. Beyond the blood vessels, normal brain parenchyma is composed of astrocytes, oligodendrocytes, neurons, and to a lesser extent neural stem cells, and immune cells such as microglia and recruited macrophages. In glioblastoma, tumor stem-like cells are a major component of the perivascular niche in addition to microglia, macrophages, and non-stem tumor cells. (Charles and Holland, 2010)

Myeloid derived cells such as microglia and macrophage are heavily studied due to the large impact the immune system has on cancer treatment outcomes, and they make up approximately 10% of cells in a malignant tumor. (Hambardzumyan et al., 2016; Wei et al., 2013) The myeloid cell signature is distinct between different types of brain tumors; glioblastoma tumors have a mixture of macrophage, activated amoeboid microglia, and ramified resting microglia. (Roggendorf et al., 1996) There is an inverse correlation between the tumor grade and the
expression of major histocompatibility (MHC) proteins such as MHC-II, which mediate immune responses, leading to a increased population of phagocytic microglia that remove extracellular debris. (Graeber et al., 2002) Local microglia migrate to the tumor area from chemokine signaling by the hepatocyte growth factor (HGF) binding to the Met receptor expressed on microglia. (Badie et al., 1999) In addition, circulating monocytes can enter the brain parenchyma and develop microglial properties. (Flügel et al., 2001)

As glioblastoma tumors develop, microglia and macrophage cells accumulate and their subsequent depletion has been shown to reduce tumor volume in a mouse model of glioblastoma. In addition, the resident microglia have been identified as being critical for tumor angiogenesis through their production of VEGF and chemokine (C-X-C motif) ligand 2 (CXCL2), among other chemokines and pro-angiogenic factors. The receptors for CXCL2 and VEGF, CXCR2 and VEGFR2, respectively, are expressed on the surfaces of endothelial cells. Interestingly, CXCL2 was a more potent inducer of angiogenesis than VEGF based on endothelial cell tube formation assays. The effect on endothelial cell proliferation between stimulation with CXCL2 or VEGFR2 was similar. (Brandenburg et al., 2016) Other important chemoattractants for microglia and macrophages include CXCL12/SDF1, glial cell-derived neurotrophic factor (GDNF), Colony Stimulating Factor-1 (CSF-1), and granulocyte-macrophage colony-stimulating factor (GM-CSF). (Hambardzumyan et al., 2016)

Myeloid cells are of particular interest for therapy as they have been shown to induce immunosuppression in glioblastoma tumors as well as aid in the evasion to anti-angiogenic therapy. A review of autopsy brain specimens found that there was an increase in macrophages
in recurrent glioblastoma patients treated with an anti-angiogenic drug compared to patients who were not treated with the same anti-angiogenic drug. In addition, examining only the patients who were treated with anti-angiogenic therapy, there was a positive correlation between the number of infiltrated macrophages and poor survival. (Lu-Emerson et al., 2013) Glioblastoma tumors express high levels of immunosuppressive cytokines such as interleukin-10 (IL-10), prostaglandin E2 (PGE-2), and transforming growth factor beta (TGF-β) that hinder current efforts to develop effective anti-cancer vaccines for patients with glioblastoma. PGE-2 is up-regulated in vitro in glioblastoma cells treated with chemotherapy or radiation, inducing immune suppression that is reversed when PGE-2 is inhibited. (Authier et al., 2015) Myeloid cells are a major source of IL-10 in glioblastoma tissues and play a role in immune suppression by inhibiting antigen presentation. (Wagner et al., 1999) TGF-β contributes to immune suppression and its expression is correlated with tumor grade. Furthermore, TGF-β has been implicated in angiogenesis and vascular remodeling as well as in supporting the glioma cancer stem-like cell population in glioblastoma. (Kaminska et al., 2013)

Glioma stem-like cells are an important component of glioblastoma tumors that largely reside in the perivascular niche. They were first described as cells with stem-like properties, such as the capacity for self-renewal, enhanced proliferation, tumor-initiating ability, and the ability to differentiate, that were isolated from brain tumors by sorting for the cell surface protein marker CD133 (prominin-1). Glioma stem-like cells are required for glioblastoma cell proliferation and maintenance in vitro and in vivo. As few as 100 CD133+ cells injected stereotactically into the NOD-SCID mouse brains are capable of tumor formation. (Singh et al., 2003, 2004) Cancer stem-like cells renew tumors in the same sense that neural stem cells participate in tissue
development. (Lathia et al., 2015) Cancer stem-like cells are regulated by intrinsic mechanisms, namely genetic, epigenetic, and metabolic processes, and extrinsic mechanisms, namely secreted factors from cells in the perivascular niche, the microenvironment such as hypoxia, and immune modulation. (Lathia et al., 2015)

Glioma stem-like cells have been implicated in resistance to radiation as the CD133$^+$ population is enriched in tumors following radiation and these cells survive radiation better than the CD133$^-$ non-stem tumor cells. Compared to their CD133$^-$ non-stem counterparts, CD133$^+$ stem-like cells have enhanced activation of the DNA damage checkpoint response after DNA damage due to radiation, and their radio-resistance can be reversed by the inhibition of Chk1 or Chk2. (Bao et al., 2006a) A potential mechanism for stem-like cell resistance may involve the upregulation of HDACs, as an in vitro study found that glioma stem-like cells have increased HDAC4 and HDAC6. (Marampon et al., 2017)

Hypoxia promotes and supports stem cell-like properties through the hypoxia inducible factors (HIF). Cancer stem-like cells express the pro-angiogenic transcription factor HIF-2$\alpha$, along with its targets, in response to hypoxic conditions to a greater extent than the non-stem tumor cells. Hypoxia and the signaling events that occur because of the harsh environment can stimulate non-stem tumor cells to form neurospheres. In addition, ectopic expression of HIF-2$\alpha$ in normoxic conditions can induce neurosphere formation and increase the CD133$^+$ fraction of cells, suggesting plasticity of tumor cells. (Heddleston et al., 2009) Further, inhibiting HIF-2$\alpha$ suppresses stem cell-like properties such as self-renewal, proliferation, and survival in vitro, and hampers tumor initiation in vivo. (Li et al., 2009) As CD133$^+$ cancer stem-like cells produce
VEGF-A and express VEGFR2, VEGF-A can act in an autocrine or paracrine manner to promote the survival of this cell population, as well as acting systemically. (Bao et al., 2006b; Hamerlik et al., 2012)

The clinical implications of cancer stem-like cells in glioblastoma tumors complicate treatment response; HIF2α expression correlates with poor survival in glioblastoma. (Li et al., 2009) In addition to resistance to radiation, cancer stem-like cells have also been implicated in the resistance to chemotherapy. A subset of quiescent tumor cells, expressing the stem cell markers sex determining region Y-box 2 (SRY, commonly known as SOX2) and Nestin, survive temozolomide therapy providing new cells for tumor re-growth. (Chen et al., 2012)

Internalization and Intracellular Trafficking

The endocytic trafficking pathway is a complex system for the internalization and trafficking of cargo that includes extracellular fluids and molecules as well as synthesized components within the cell. Through complex regulation and redundancy, the mechanisms of endocytic trafficking of cargo are still being elucidated through active research. (Doherty and McMahon, 2009) Due to the possible outcomes of molecules that are trafficked through the cell, endocytic trafficking could play an important role on the effect of therapeutic molecules.

Several types of internalization mechanisms exist including clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin/caveolin-independent endocytosis that rely on actin
polymerization, such as macropinocytosis. Each cell type may have varying proportions of these different endocytosis mechanisms functioning based on specific cell functions and the microenvironment. Clathrin-mediated endocytosis has been described to a greater extent than the other methods of internalization, although recent advances and improved techniques are shedding more light on other mechanisms of endocytosis. (Doherty and McMahon, 2009)

Clathrin-coated pits have a size limitation of approximately 200 nm in diameter due to the rigid three-dimensional structure (McMahon and Boucrot, 2011). Caveolin endosomes are even more limited in size and are typically no larger than 60-80 nm in diameter. (Parton and Simons, 2007) Macropinocytosis, however, is not limited in size; macropinosomes are a heterogeneous mixture of vesicles that are between 0.2 µm and 5 µm in diameter. (Hewlett et al., 1994)

Macropinocytosis differs from clathrin- and caveolin-mediated internalization methods for several reasons in addition to vesicle size. Macropinocytosis is not initiated by cargo binding to receptors but instead is activated by growth factor signaling such as EGF and PDGF that stimulate actin polymerization to produce plasma membrane ruffles. (Haigler et al., 1979; Kerr and Teasdale, 2009; Lim and Gleeson, 2011; Nakase et al., 2015) Macropinocytosis non-selectively internalizes extracellular fluids from actin protrusions falling back onto the cell and fusing with the membrane to form a macropinosome. Due to the non-specific nature of the cargo that is internalized by macropinocytosis, fluid-phase markers that are rapidly and non-selectively internalized can be used to visualize and quantify macropinosomes. The fluid-phase marker that is commonly used for measuring macropinocytosis through monitoring its cellular uptake is tetramethylrhodamine-conjugated 70kDa dextran (TMR-70-kDa-dextran). (Commissio et al.,
2014) Macropinocytosis is regulated by small GTPases such as Rac1 and Cdc42 that hydrolyze GTP (guanosine triphosphate). (Garrett et al., 2000) Macropinocytosis can be pharmacologically blocked by 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), an inhibitor of the Na(+)/H(+) ion exchange pump that is critical for reducing macropinocytosis-induced local acidity, which would otherwise inhibit GTPase activity. (Koivusalo et al., 2010)

The membranes of macropinosomes containing internalized cargo can fuse with the membrane of early endosomes and other endocytic compartments that combine cargo from various internalization mechanisms. The cargo within early endosomes can be trafficked to a recycling compartment or the lysosome. (El-Sayed and Harashima, 2013; Lim and Gleeson, 2011)

Trafficking to the lysosome may be regulated, as has been shown for the targeted degradation of EGFR when it is internalized by a clathrin-independent mechanism but not when it is internalized by clathrin-mediated endocytosis. (Sigismund et al., 2008) The regulation of cargo degradation could be targeted therapeutically.

Immunofluorescence confocal microscopy is a common method for visualizing and quantifying endosomal compartments using primary antibodies that bind to endosomal markers, such as early endosome antigen 1 (EEA1), ras-related protein Rab-4A (Rab4), Rab11, and lysosomal associated membrane protein 1 (LAMP1) for marking early endosomes, fast recycling endosomes, slow recycling endosomes, and late endosomes, respectively. (Watson et al., 2005)
Macropinocytosis could be a method for cells to internalize therapeutic drugs, including large molecules such as the humanized monoclonal antibody bevacizumab (150 kDa). One report indicated that human IgG was detectable in tumor lysates of orthotopic glioblastoma xenograft tumors that had been propagated in nude mice administered bevacizumab. (Burkhardt et al., 2012) However, this was based on western blotting of whole tumor lysates and, thus, did not differentiate between the presence of the bevacizumab in the perivascular space and the tumor-associated vessels.

Macropinocytosis could be a clinically significant pathway that potentially affects treatment outcomes. Macropinocytosis has been shown to be a mechanism by which Ras-transformed cells and starving cells engulf extracellular proteins and then lysosomally degrade the proteins, thereby promoting their own proliferation and survival. (Commissio et al., 2013; Kamphorst et al., 2015) Macropinocytosis may be involved in the pathogenesis of cancer metastasis as it has been shown to play a role in migration through the shuttling of integrins from the trailing edge to the leading edge of the cell. (Paul et al., 2015)

**Autophagy and Cell Survival**

Autophagy is an important process that normally occurs at a basal level in cells for the maintenance of organelles and to rid the cell of misfolded proteins. This process can be non-selective since the autophagosome sequesters bulk cytoplasmic contents during its formation. (Mizushima, 2005) At the induction of autophagy, the double-membraned phagophore elongates
and envelopes around cytoplasmic contents, thereby encapsulating them into an autophagosome. Lysosomes fuse with the autophagosome to form autolysosomes that then degrade the inner membrane and cytoplasmic contents, generating amino acids, glucose, and other basic building blocks that are then released back into the cytoplasm. (Mizushima et al., 2010)

The survival role of autophagy has been attributed in large part to the production of amino acids. Specifically, glutamine is considered to be one of the more important amino acids produced through autophagy as glutamine supplementation could reverse cell death caused by the inhibition of autophagy. (Guo et al., 2016; Strohecker et al., 2013) In response to stress caused by nutrient deprivation, autophagy is upregulated – or induced – as a survival mechanism. (Mizushima, 2005; Singh et al., 2017) Cultured cells that depend on growth factors can survive for several weeks without the necessary growth factors but will die immediately following inhibition of autophagy. (Lum et al., 2005) Thus autophagy can be induced by growth factor starvation.

The roles of basal and induced autophagy are very different; basal autophagy maintains the integrity of cytoplasmic organelles and proteins while induced autophagy produces amino acids essential for cellular survival. (Mizushima, 2005, 2007) In cancer, basal autophagy is elevated, suggesting a greater need for cancer cells to remove toxic waste from rapidly proliferating tumor cells as well as the increased need for an amino acid supply to support the high metabolic demands of tumors. (Yang et al., 2011b)
Autophagy has been shown to act as a pro-survival mechanism as well as promoting cell death. Evidence for cell death induced by autophagy comes from several reports including a study showing a reduction in non-apoptotic cell death by knock-down of ATG7 and beclin1 in cells treated with a caspase inhibitor and a study that shows enhanced autophagy-associated cell death in Bax/Bak knockout mice treated with staurosporine. (Shimizu et al., 2004; Yu et al., 2004)

In addition to nutrient deprivation, hypoxia can induce pro-survival autophagy in normal and cancer cells, including glioblastoma cells, through targets of the hypoxia-induced HIF-1 transcription factor and the autophagy protein ATG9. (Abdul Rahim et al., 2017; Bellot et al., 2009; Karantza-Wadsworth et al., 2007; Zhang et al., 2008) Further, the anti-angiogenic therapy bevacizumab has been reported to promote autophagy-induced vascular mimicry by glioma stem-like cells. (Wu et al., 2017)

A recent study found that glioblastoma cells cultured in vitro are more resistant to the alkylating agent temozolomide, used clinically as a standard of care for glioblastoma patients, due to the upregulation of autophagy. (Hombach-Klonisch et al., 2017) Initially it was thought that temozolomide induces cell death by autophagy but further analysis revealed that pro-survival autophagy was induced. (Kanzawa et al., 2004; Katayama et al., 2007) To further support the concept that autophagy plays an important role in temozolomide resistance, several studies have shown that inhibiting autophagy with chloroquine, an anti-malarial drug that prevents lysosomal acidification that is necessary for the production of amino acids and other basic building blocks, sensitizes glioblastoma cells to temozolomide in a mouse model of glioblastoma. This increased sensitivity to temozolomide chemotherapy with concomitant autophagy inhibition appears to
only occur when autophagy is inhibited at the later stages, such as with chloroquine or knockdown of beclin1, as inhibition of early stages of autophagy with 3-methyladenine (3-MA) did not sensitize glioblastoma cells to undergo apoptosis with arsenic trioxide treatment. (Golden et al., 2014; Hori et al., 2015; Li et al., 2015)

Autophagy, especially when combined with macropinocytosis, is closely linked to cell metabolism. Alterations in metabolism is an emerging hallmark of cancer. Proliferating cancer cells must gain biomass and energy to sustain tumor growth through altered methods for obtaining glucose, glutamine, amino acids, and other building blocks and this can occur through macropinocytosis and other processes. If extracellular nutrients are low, cancer cells must rely on the breakdown of internal organelles and cytoplasmic contents to survive through the process of autophagy. It is worth noting that autophagy does not provide new biomass for proliferating tumors but could be responsible for resistance to treatment. (Flavahan et al., 2013; Kamphorst et al., 2015; Pavlova and Thompson, 2016)

Significance

The overall goal of our studies is to identify whether the outcome of monoclonal antibody therapy is determined by the macropinocytosis and endocytic trafficking status of cancer stem-like cells that are found in the perivascular niche and whether poor responses to these therapies can be corrected through targeting these pathways. Immunotherapies have proven to be an extremely promising therapeutic approach in some cancer patients. However, the anticipated
impact of this new class of biologic therapeutics on patient care and potential cure is limited as a large number of patients do not show an effective response and, in the case of monoclonal antibody therapies, most patients who show an initial response subsequently relapse. Identification of targetable mechanisms underlying intrinsic and acquired resistance to these therapies would have a major impact on patient care.

The experimental model that we have selected for these studies is the use of the therapeutic monoclonal antibody bevacizumab that is used for treatment of recurrent glioblastoma. Glioblastoma is an extremely heterogeneous disease; however, the majority of patients with recurrent glioblastoma are now treated with bevacizumab (Ahluwalia and Gladson, 2010; Braghroli et al., 2012; Robles Irizarry et al., 2012) due to bevacizumab-induced reduction in cerebral edema and an increased PFS. Thus, adequate numbers of patient-derived xenograft models representing response to therapy or the lack of response are available.

Approximately 20% of patients with recurrent glioblastoma have intrinsic resistance to bevacizumab (Lu-Emerson et al., 2015; Nowosielski et al., 2014) and most patients who show an initial response subsequently exhibit acquired resistance. (Lu-Emerson et al., 2015; Nowosielski et al., 2014) Also, it has been reported that bevacizumab suppresses tumor proliferation, as measured by positron emission tomography (PET) imaging, in responding patients. (Schwarzenberg et al., 2014) However, internalization via the non-selective process of macropinocytosis (Lim and Gleeson, 2011) and selective trafficking of monoclonal antibodies by a tumor cell population, including perivascular cancer stem-like cells, has not been considered in the development of the current understanding of bevacizumab resistance in recurrent
glioblastoma. Bevacizumab was originally developed as an anti-angiogenic therapy and a number of studies have focused on its effects on the vasculature. Our data are consistent, however, with the increasing evidence that bevacizumab can directly affect tumor cell biology and can act directly on xenograft perivascular tumor cells, inducing autophagy in vitro and in vivo. (Liang et al., 2015; Müller-Greven et al., 2017)

Specific Aims

We aim to determine whether the macropinocytosis-associated endocytic trafficking status of cancer stem-like cells plays a critical role in shaping the responses to therapeutic monoclonal antibodies. Treatment of patients with recurrent glioblastoma with bevacizumab (Avastin®), a humanized anti-VEGF-A monoclonal antibody, has been shown to extend the PFS in a significant number of patients but this does not result in a significant extension in OS in most of these patients due at least in part to the development of acquired resistance. The mechanisms underlying intrinsic and acquired resistance have not been identified and the role of cancer stem-like cells has not been explored although they are thought to be responsible for glioblastoma resistance to chemotherapy and radiation therapy. (Bao et al., 2006a, 2006b; Cheng et al., 2010; Lathia et al., 2012; Schonberg et al., 2014)

Our current findings suggest that bevacizumab can directly affect tumor cell biology and can act directly on perivascular cancer stem-like cells, inducing autophagy in vitro and in vivo. (Müller-Greven et al., 2017) These findings support a potentially novel mechanism in which alterations
in the intracellular trafficking of bevacizumab may lead to (i) bevacizumab being targeted largely to the lysosome thereby preventing clearance of VEGF-A in the immediate vicinity of cancer stem-like cells and promoting an unabated proliferative response associated with intrinsic resistance; and (ii) the emergence of a compensatory autonomous survival mechanism (autophagy) induced by bevacizumab depletion of VEGF-A that in the cancer stem-like cells ultimately drives resistance (acquired resistance). A corollary of these hypotheses is that co-administration of agents that inhibit endosome maturation to lysosomes and enhance recycling, or convert a pro-survival autophagy into a cytotoxic response, would improve therapeutic outcomes in glioblastoma. The potential clinical relevance of the macropinocytic process in bevacizumab responsiveness is suggested by the finding of a high representation of alterations in genes associated with macropinocytosis and trafficking pathways in preliminary comparisons biopsies from glioblastoma patients who either responded to bevacizumab therapy or did not.

To test this model, we used cancer stem-like cell isolates from patient-derived xenograft (PDX) tumors for in vitro analysis and PDX and syngeneic immune-competent mouse models of glioblastoma for in vivo analysis to investigate in Aim 1 whether cancer stem-like cells internalize humanized monoclonal antibodies, such as bevacizumab, via macropinocytosis and enter the endocytic pathway, potentially affecting cancer stem-like cells survival. In Aim 2, we investigated whether bevacizumab induces autophagy in vitro and in vivo in perivascular tumor cells in the PDX and syngeneic immune-competent mouse models of glioblastoma.

**Aim 1: Determine if bevacizumab is internalized and trafficked by perivascular CSLCs.**
There is evidence that alterations of the endocytic pathway occur in cancer and affect survival. (Johnson et al., 2015; Mellman and Yarden, 2013; Mosesson et al., 2008; Peiris-Pagès et al., 2015; Yang et al., 2011a; Yu et al., 2015) Among the several cell types present in the perivascular niche, cancer stem-like cells (CSLCs) are known to be important players in tumor initiation, maintenance, and resistance to therapy. (Lathia et al., 2015) In Aim 1, we investigated whether cancer stem-like cells internalize humanized monoclonal antibodies, such as bevacizumab, and whether the trafficking of these antibodies affect cancer stem-like cell survival.

**Aim 2: Determine if internalized bevacizumab induces autophagy in perivascular CSLCs.**

Considering that cancer stem-like cells degrade a fraction of internalized bevacizumab, it is plausible that the amino acids generated from degradation provide basic building blocks for the cells and thus a survival advantage. Bevacizumab also binds strongly to VEGF-A that can lead to apparent growth factor deprivation, as the VEGFR2 receptor is not activated to produce growth factor signaling in the cell. We have shown that glioblastoma cancer stem-like cells grown in vitro undergo autophagy and have reduced cytotoxicity when treated with bevacizumab; supporting the hypothesis that bevacizumab can promote survival. We also investigated whether bevacizumab induces autophagy in vivo in perivascular tumor cells in the patient-derived xenograft mouse model of glioblastoma. In vivo confirmation of our in vitro findings supports the translational significance of our findings and potentially explain, at least partly, the variation in clinical response observed in patients.
Patients with glioblastoma have a dismal prognosis (median survival <15 months). Our expectation is that the results of the studies will shift the research paradigm by emphasizing the role of cancer-associated alterations in sub-cellular trafficking pathways in bevacizumab responsiveness.
CHAPTER 2

MATERIALS AND METHODS

 Cells
Normal brain ECs (isolates 422 and 376) were purchased from Cell Systems and tumor-associated ECs (TECs) were isolated and propagated from primary human GBM tumors in media recommended by Cell Systems for brain ECs (Huang et al., 2012). Normal brain ECs were used during the first 8 passages, and the cell type verified by positive staining for von Willebrand factor (vWF) and tube formation on Matrigel™ or collagen gels. (Huang et al., 2012) TECs were also used during the first 8 passages, sorted every two passages for CD31 or CD105 positivity,
and the cell type verified as described. (Huang et al., 2012) CD133+ cells and the matched (paired) CD133-negative (non-stem) tumor cells were isolated from two human GBM xenograft tumors (08-387 and 4121). CD133+ GBM cells were propagated in suspension in Neurobasal A media (NBM) with B27 and N2 supplements (Life Technologies) and with EGF (10 ng/ml) and (basic fibroblast growth factor) bFGF (5 ng/ml), as described. (Eyler et al., 2008) The matched non-CD133+ (non-stem) tumor cells were propagated in DMEM media with 10% FBS, conditions that induce differentiation of the CD133+ cells. (Eyler et al., 2008) MM.1R human myeloma cells were purchased from the ATCC. MM.1R human myeloma cells were propagated as described. (Zheng et al., 2013) Authentication of cell identity was performed using short tandem repeat (STR) profiling.

**Reagents**

The following reagents were purchased: 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) (Sigma-Aldrich #A3085); lysine-fixable tetramethylrhodamine(TMR)-70-kDa-Dextran (Molecular Probes #D1818); and biotinylated recombinant human-VEGF165 (ACRO Biosystems #VE5-H8210). The following antibodies were purchased: Alexa-Fluor®-488-phalloidin, Alexa-Fluor®-488- and Alexa-Fluor-633®-anti-human IgG, Alexa-Fluor®-594-anti-rabbit IgG, Alexa-Fluor®-594-anti-mouse IgG, and Alexa-Fluor®-647-Streptavidin (Molecular Probes); mouse or rabbit anti-vWf (Abcam #ab68545, Millipore #AB7356), rabbit anti-Rab4 (Thermo Scientific #PA3-912), rabbit anti-LAMP1 (Abcam #ab24170), mouse anti-SOX2 (R&D Systems #MAB2018), rabbit-anti-Iba1 (Wako #019-19741), rabbit anti-human FcRn (Santa Cruz #sc-66892), goat anti-FcγRIIB (R&D Systems #AF1330), mouse anti-LC3 (Proteintech #10397-1-AP, Abcam #ab25631),
mouse Alexa Fluor® 488-anti-human nuclear antibody (EMD Millipore #MAB1281A4), goat anti-actin (Santa Cruz #sc-1615), mouse mAb anti-VEGFR2 (Cell Signaling #9698 clone D5B1), rabbit anti-phosphotyrosine (Millipore #05-1050), rabbit mAb anti-cleaved caspase-3 (Cell Signaling #9664), rat anti-Ki67-Alexa-647 (BioLegend #151206), sheep anti-mouse CD44 (R&D Systems #AF6127), and goat anti-rat IgG-Alexa-488 (ThermoFisher Scientific #A-11006). Control human IgG was purchased (Sigma Aldrich #I4506). Bevacizumab was a gift (Cleveland Clinic pharmacy).

**Expression of FcγRIIB in myeloma cells**

FcγRIIB cDNA was purchased (EX-ZO435-Lv105, GeneCopoeia) and a lentivirus vector created and used to transduce MM.1R myeloma cells. (Zheng et al., 2013)

**Animal Studies**

All studies were performed with the approval of the Animal Use and Care Committee of the Cleveland Clinic, Mayo Clinic or Emory University School of Medicine. The CD133⁺ orthotopic GBM model, female nude mice (4-6 weeks of age) were purchased (Taconic) and injected intracerebrally with 15,000 CD133⁺ GBM cells using an established procedure. (Eyler et al., 2008) At 14 days, monitoring of mouse brains for luciferase activity was initiated and continued every 3 days at the Cleveland Clinic Bioluminescent Imaging Facility. When a predetermined amount of luciferase activity was detected, daily intraperitoneal (i.p.) injection of bevacizumab or hIgG was initiated and continued for 5 days, followed by euthanasia and harvesting of the brains. The patient-derived GBM xenograft (PDX) tumors (G39, G59, G44) were established intracerebrally in athymic nude mice as described. (Carlson et al., 2011)
Treatment with bevacizumab was initiated on day 13 (PDX G39), day 20 (G44), or day 28 (PDX G59) post-injection and given twice weekly by i.p. injection (5mg/kg) until euthanasia at the detection of neurologic signs, followed by brain harvest. (Carlson et al., 2011) Frozen blocks randomly selected from three bevacizumab-treated and two or three vehicle-treated tumor-bearing brains from each of the three PDX tumors were sectioned. One vehicle-treated tumor from G39 was excluded due to differences in processing. For the somatic gene transfer GBM model, six to eight-week-old Ntv-a/ink4a-arf−/− mice were used to generate GBM tumors via introduction of RCAS-PDGF-B-HA, as described. (Hambardzumyan et al., 2009) Rat anti-mouse-VEGF-A IgG (Basu et al., 2008) (BioLegend clone 2G11-2A05) (5 mg/kg i.p., 2X/week) was administered once the tumors were established (4 weeks post-introduction of RCAS-PDGF-B-HA) and continued for two weeks, followed by euthanasia, brain harvest and fixation as above.

**Immunofluorescence**

CD133+ GBM cells were plated overnight on chamber slides coated with 20 µg/ml laminin (R&D Systems #3400-00-01) in neural basal media (NBM) without addition of EGF or bFGF. Maintenance of the stem-cell phenotype was verified by SOX2 expression. (Fael Al-Mayhani et al., 2009; Gangemi et al., 2009) The following day, a clinically relevant concentration (250 µg/ml) (Klettner and Roider, 2008) of bevacizumab was added to the media. After incubation (37°C, 5% CO2) for 5 min, the cells were washed and fixed, or fresh media was added and the cells incubated for the times indicated. To detect internalized bevacizumab, cells were fixed with 4% buffered-paraformaldehyde, blocked, and reacted with Alexa-488-anti-human IgG, then mounted in media containing DAPI nuclear stain (Vector Lab #280772).
Double-label immunofluorescent analysis was performed as described previously. (Huang et al., 2012; Ryan et al., 2010) Double-label immunofluorescence was visualized and photographed using Leica laser confocal microscopes (Leica-SP5 and Leica-TCS-SP2-AOBS) and a Leica-DM5500B upright microscope. Immunofluorescent conditions were assayed in triplicate and 10 fields per replicate analyzed for co-localization. Analysis of co-localization was carried out as described using ImageJ software with the JACoP-plugin. (Bolte and Cordelières, 2006; Watson et al., 2005) For each field, only p values indicating a >95% certainty of co-localization were utilized in determining the Mander’s coefficient. (Bolte and Cordelières, 2006; Watson et al., 2005) Controls included secondary antibody alone.

For immunofluorescence analysis of frozen sections, tissue sections (8-µm) were fixed in 4%-paraformaldehyde, blocked, reacted with the primary antibodies overnight, washed, reacted with the secondary fluorescent-conjugated antibodies, followed by cover slipping with mounting media containing DAPI nuclear stain, as described. (Burgett et al., 2016) Sections were viewed and photographed using a Leica laser confocal microscope as described above.

**Cytotoxicity Assay**

This assay was performed according to the manufacturer’s instructions (CytoTox-Fluor™ Cytotoxicity Assay, Promega).

**MTT Assay**
This colorimetric assay measures metabolic rate, a measure of proliferation, by assessing the reduction of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) to the purple formazan by NAD(P)H-dependent oxidoreductase enzymes released from cells and was performed according to the manufacturer’s instructions (CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT), Promega).

**ELISA Assays**

VEGF-A, VEGF-C and placental growth factor (PGF) were measured using VEGF-A&C (RayBiotech) and PGF (R&D-Systems) ELISA Assay Kits as recommended by the manufacturer.

**ELISA Assay for Bevacizumab**

Recombinant-human-VEGF-A165 (100 ng/ml in 50 µl of phosphate-buffered saline (PBS); BioVision #4363) was coated on 96 well Immulon H2B plates (Thermo Fisher Scientific #3455) overnight at 4°C. The following day the wells were washed 3X with PBS, blocked with 200 µl of 2% BSA/PBS (2 hours, 22°C), followed by addition of 200 µl of various dilutions of media from the bottom chamber of the transcytosis assay containing bevacizumab and incubated overnight at 4°C. The next day the wells were washed 3X with PBS, horse radish peroxidase (HRP)-conjugated-anti-human IgG was added (200 µl of a 0.5 µg/ml solution in PBS) and incubated (1 hour, 22°C), washed 3X with PBS, followed by addition of 100µl TMB substrate (3,3’,5,5’-tetramethylbenzidine) (Thermo Fisher Scientific #34028) (2 min, 22°C) and 2M sulfuric acid to stop the reaction, as described. (Schneider et al., 2014) The absorbance was read at 450 nm in an ELISA Plate Reader. All samples were analyzed in replicates of three. The
absorbance readings were compared to a standard curve of bevacizumab (0.24 to 15.6 ng/ml) that was immobilized on the plate in replicates of three.

**Transcytosis Assay**

A standard transcytosis assay for brain ECs was performed as described. (Monaghan-Benson and Wittchen, 2011; Weksler et al., 2005) Briefly, ECs (isolate 422 or 376) or TECs were plated as a confluent monolayer on collagen coated 0.4-µm-pore filters in Boyden chambers in regular media. After 3 days, 70-kDa-FITC-Dextran (Molecular Probes, #D1823) (FITC: fluorescein isothiocyanate) or bevacizumab was added to the confluent monolayer of ECs or TECs in the wells, and the fluorescence in the bottom chamber quantitated over time.

**Statistical Analysis**

All statistical analyses were performed or overseen by, Amy S. Nowacki, a biostatistician in the department of Quantitative Health Services (QHS) at the Cleveland Clinic. The test used for each experiment is stated in the Figure Legends. A p-value < 0.05 was considered significant.
RESULTS

Trafficking of bevacizumab in glioblastoma does not involve significant transcytosis across normal brain endothelial cells or endothelial cells isolated from glioblastoma tumors

Transcytosis (reviewed in (Preston et al., 2014)) across endothelial cells (ECs) is regulated in normal brain, but potentially could be abnormal in endothelial cells isolated from glioblastoma tumors (TECs). Using a standard in vitro transcytosis assay for normal brain ECs (Preston et al., 2014), we compared transcytosis of bevacizumab across monolayers of normal brain ECs and TECs. Quantitation of the bevacizumab in the lower chamber by enzyme-linked immunosorbent assay (ELISA) showed that ~30% of bevacizumab was transcytosed across both normal brain ECs and TECs over two hours (Figure 1A). There was a two-fold larger permeability coefficient
for 70-kDa-FITC-Dextran in TECs as compared to the normal brain ECs (Figure 1B). Both normal brain ECs and TECs internalized bevacizumab over 30 min (Figure 1C&D).

Collectively, these data suggest that transcytosis of bevacizumab is not enhanced in TECs, supporting the concept that bevacizumab gains access to the perivascular tumor space in glioblastoma due to alterations in the blood-brain barrier.
Figure 1. Bevacizumab is transcytosed across normal brain ECs and TECs. Normal primary brain ECs or ECs isolated from GBM (TECs) were plated onto collagen in regular media. A&B, At three days post-plating the confluent monolayer was treated with bevacizumab, and at the indicated times media was collected from the lower chamber and subjected to an ELISA assay for human IgG to quantitate transcytosis (A). Alternatively, at three days post-plating the brain ECs or TECs was treated with 70-kDa-FITC-Dextran and the permeability of the monolayer determined by measurement of the fluorescence in the bottom chamber over time and expressed as the volume crossing the monolayer (B). In A&B, open diamonds denote absence of ECs on the filter; closed triangles denote the average of two isolates of normal brain ECs; and closed circles
denote TECs. C&D, The day following plating, bevacizumab was added to the media and at 30 min the cells were washed, fixed, reacted with Alexa-488-anti-human IgG, followed by DAPI nuclear stain and microscopy. Scale bars denote 20µm.
Bevacizumab gains access to the perivascular space and is internalized predominantly by perivascular SOX2+/CD44+-tumor cells in orthotopic xenograft and syngeneic mouse models of glioblastoma

To determine whether bevacizumab can gain access to the perivascular space in glioblastoma, we used a mouse model in which bevacizumab or a control hIgG was administered (i.v., 5 d) to mice with established glioblastoma tumors. Bevacizumab and hIgG were detected in frozen sections of the brain by labeling with an Alexa-488-anti-human-IgG (green) and their location assessed by co-staining with anti-von Willebrand factor (vWf), a marker of ECs, and Alexa-594-conjugated secondary antibody (red) together with nuclear staining using DAPI (blue). Both bevacizumab and the control hIgG were detectable in the perivascular space of the orthotopic glioblastoma tumors (Figure 2A&B). Very little bevacizumab or hIgG was detected in the perivascular space of uninvolved brain from the mouse model (Figure 2A&B), consistent with prior studies indicating that minimal amounts of hIgG gain access to the perivascular tissue when the blood brain barrier (BBB) is intact. (Yu et al., 2011)
Figure 2. Bevacizumab gains access to the perivascular tumor space and is internalized by tumor cells when administered to an orthotopic xenograft model of GBM. Luciferase-labeled CSLCs (15,000) were injected intracerebrally into the nude mouse brain, at 14 days bioluminescent
imaging was initiated and once the tumor reached a predetermined size bevacizumab or IgG administration was begun and continued for 5 days. The mice were then euthanized, the brains harvested, fixed in buffered formalin and embedded in paraffin (A), or fixed in 4% paraformaldehyde, immersed in 20% sucrose and frozen (B). Sections were reacted with Alexa-488-anti-human IgG (green), and anti-vWF IgG, followed by an Alexa-594-secondary antibody (red). Area of tumor from mouse administered bevacizumab or IgG is shown along with adjacent normal mouse brain. Arrows denote ECs (blood vessels) and arrowheads denote bevacizumab. Scale bars denote 10µm.
This analysis further indicated that bevacizumab is internalized by perivascular tumor cells in the glioblastoma xenografts (Figure 2A&B). Using serial sections of xenograft tumors, we verified that the perivascular cells were human tumor cells by double-labeling with a monoclonal antibody anti-SOX2 and a rabbit antibody to human nuclei (Figure 3). The relative distribution of bevacizumab in the SOX2+ cells versus activated microglia/macrophages was determined using intracerebral glioblastoma xenograft tumors obtained from mice in which the tumor was established and then bevacizumab administered until euthanasia. Sections of the tumors were stained with antibodies to SOX2, a marker of cancer stem-like cells, and ionized calcium-binding adapter molecule 1 (Iba1), a marker of activated microglia and macrophages (Ahmed et al., 2007; Gangemi et al., 2009), and the fluorescent intensity of bevacizumab quantified. Although both perivascular SOX2+ cancer stem-like cells and Iba1+ activated microglia and macrophage cells internalized bevacizumab, the bevacizumab-intensity was ~2.5-fold higher in the perivascular SOX2+ cells than perivascular Iba1+ cells (Figure 4A&B). On measuring the distance of SOX2+ and Iba1+ cells from the nearest blood vessel, we did not find a difference in proximity (Figure 4C), consistent with the known localization of both cell types to the perivascular niche (1), although we did find that the mean number of SOX2+ cells was higher than the mean number of Iba1+ cells within a 25-μm-radius of the nearest blood vessel (Figure 4D).
Figure 3. Confirmation of cancer stem-like cell marker SOX2 in vivo in an orthopic xenograft model of GBM. Perivascular human tumor cells detected with rabbit anti-human nuclei and mAb anti-SOX2 in the xenograft model of GBM from A&B. Sections were double-labeled with mAb anti-SOX2 (2 µg/ml) and rabbit anti-human nuclei (5 µg/ml, Millipore cat# MAB1281A4), followed by Alexa-488 and Alexa-594 secondary antibodies, respectively, and DAPI nuclear stain.
Bevacizumab gains access to the perivascular tumor space and is internalized predominantly by perivascular SOX2+/CD44+ tumor cells in a PDX xenograft mouse model of GBM. PDX GBM tumors (G39 or G59) were injected intracerebrally into the nude mouse (300,000 cells), and treatment with bevacizumab (5 mg/kg, i.p., 2X/week) or placebo initiated on day 13 (G39) or day 28 (G59). Treatment was continued until the development of neurologic
signs at day 23 (G39) or day 50-77 (G59), followed by euthanasia, brain harvest, freezing in OCT media, and storage at -80°C. Sections from three mouse tumors from each of the two GBM xenografts treated with bevacizumab were reacted with Alexa-488-anti-hIgG (green) and anti-SOX2 (2.5 μg/ml) or anti-lba1 (0.5 μg/ml) antibody (red), and rabbit or mouse anti-vWF (5 μg/ml or 2 μg/ml, respectively) antibody (blue), followed by Alexa-594-conjugated- and Alexa-647-conjugated secondary antibodies and DAPI nuclear stain (A). Area of tumor from mouse administered bevacizumab shown. Arrows denote bevacizumab in SOX2+ tumor cells or in lba1+ activated microglia/macrophages (A). Scale bars denote 10-μm (A). Bevacizumab fluorescent intensity in SOX2+ or in lba1+ perivascular cells was quantitated as the mean signal intensity/field area and as the mean signal/cell using ImageJ in the six bevacizumab-treated G39 and G59 GBM xenograft tumors (B). The mean distance of bevacizumab-positive SOX2+ or lba1+ perivascular cell from the nearest EC was quantitated using ImageJ on the same six xenograft tumors (C). The mean number of SOX2+ or lba1+ cells within a 25-μm radius of vWF-positive blood vessels was quantitated on the same six xenograft tumors (D).
These observations were confirmed using an established immunocompetent mouse model in which glioblastoma tumors were induced by PDGF-B and then treated for two weeks with rat anti-mouse-VEGF-A IgG followed by euthanasia. The cell-surface glycoprotein CD44 marks cancer stem-like cells in this model (Pietras et al., 2014) and we found that they also express SOX2 (Figure 5). Tumor sections were stained with antibodies to CD44 or Iba1, as well as vWf, and the fluorescent intensity of rat anti-mouse-VEGF-A IgG quantified. We found that both perivascular CD44+ and Iba1+ cells within a 25-μm radius of blood vessels internalized anti-mouse-VEGF-A. The rat anti-mouse-VEGF-A-intensity was ~5-fold higher in the CD44+ perivascular cells as compared to that in the Iba1+ perivascular cells (Figure 6A&B). The distance of CD44+ perivascular cells from the nearest blood vessel was slightly less than that for Iba1+ cells (Figure 6C).
Figure 5. **Confirmation of cancer stem-like cell marker SOX2 in vivo in a syngeneic mouse model of GBM.** Perivascular CD44+ cells also mark with mAb anti-SOX2 in the immune competent PDGF-B-induced mouse model of GBM. The syngeneic somatic gene transfer model of GBM was generated by intracerebral injection of four 6- to 8-week-old Ntv-a/ink4a-arf/- mice with RCAS-PDGFB-HA. After four weeks, treatment with rat anti-mouse VEGF-A IgG (5 mg/kg, i.p., 2X/week) was initiated and continued for two weeks, followed by euthanasia, brain harvest, fixation in 4% paraformaldehyde, immersion in sucrose and freezing (-80°C). Sections were reacted with sheep anti-mouse CD44 (5 µg/ml; R&D Systems cat#AF6127) and with the monoclonal antibody anti-SOX2 (2 µg/ml), followed by Alexa-488 and Alexa-594 secondary antibodies, and DAPI nuclear stain.
Figure 6. Bevacizumab gains access to the perivascular tumor space and is internalized predominantly by perivascular SOX2+/CD44+ tumor cells in a syngeneic mouse model of GBM.

Tumors were established as described in Figure 5. Tumor bearing sections (5-µm) were reacted with Alexa-488-anti-rat IgG (green), sheep anti-mouse CD44 (5 µg/ml) or rabbit anti-Iba1 (red), and rabbit or mouse anti-vWF, respectively (as above) (blue), followed by Alexa-594 and Alexa-647-conjugated secondary antibodies, and DAPI nuclear stain (A). Rat anti-mouse-VEGF-A fluorescent intensity in CD44+ or in Iba1+ perivascular cells was quantitated as the mean signal...
intensity/cell using ImageJ in the four mice (B). The mean distance of rat anti-mouse-VEGF-A-positive CD44+ or Iba1+ perivascular cell from the nearest EC was quantitated using ImageJ on the same four tumors (C). Statistical analyses in panels B&C: linear mixed model.
Bevacizumab induces autophagy in perivascular tumor cells in the xenograft model of glioblastoma

We then determined whether there were differences in apoptosis of the perivascular tumor cells in the bevacizumab- and placebo-treated xenograft tumors by double labeling for cleaved caspase-3 and SOX2. We did not find a significant difference in the numbers of apoptotic SOX2+ cells in bevacizumab-treated as compared to placebo-treated tumors (p = 0.50) (Figure 7A&B). To determine whether bevacizumab induced autophagy, we examined the colocalization of LC3 puncta with lysosomal associated membrane protein 2 (LAMP2). (Galluzzi et al., 2015) We found a significantly higher number and greater area of LC3 puncta colocalized with LAMP2 in the bevacizumab-treated as compared to the placebo-treated tumors (Figure 8A-C), suggesting that bevacizumab therapy of glioblastoma xenografts induces autophagy in vivo.
Figure 7. Bevacizumab treatment of an orthotopic xenograft model of GBM does not induce apoptosis. Sections of xenograft GBM tumor from the G39 and G59 PDX tumors treated with bevacizumab or placebo were reacted with rabbit anti-cleaved caspase-3 (0.2 µg/ml) (green) and mAb anti-SOX2 (2.5 µg/ml) (red) antibodies, followed by Alexa-488 and Alexa-594-conjugated secondary antibodies, DAPI nuclear stain and confocal microscopy. Representative photographs of tumor are shown (A). Quantitation of the number of double-labeled cleaved caspase-3 and SOX2+ cells in five representative fields/tumor (B). Data are graphed as Box and Whisker plots. Statistical analyses panel B: linear mixed model.
Bevacizumab treatment of an orthotopic xenograft model of GBM induces autophagy.

Sections of xenograft GBM tumor from the G39, G59 and G44 PDX tumors treated with bevacizumab or placebo were reacted with anti-LC3 (2ug/ml) and anti-LAMP2 (4ug/ml) antibodies, followed by Alexa-488 and Alexa-594-conjugated secondary antibodies, DAPI nuclear stain and confocal microscopy. Representative photographs of tumor are shown (A). Quantitation of the number of LC3-LAMP2-positive puncta/cell in 10 representative fields/tumor (B); the adjusted means are 2.1 versus 0.8 for bevacizumab-treated versus
placebo-treated tumors, respectively (p=0.02). Quantitation of the area of LC3-LAMP2-positive puncta/cell in 10 representative fields/tumor (C); the adjusted means are 0.35 versus 0.12 for bevacizumab-treated versus placebo-treated tumors, respectively (p=0.02). Data are graphed as Box and Whisker plots; the green dots represent the adjusted means. In panel B, two data points in the bevacizumab-treated group (10.36 and 14.73) were removed for graphing purposes, but were included in the statistical analysis. In panel C, one data point in the bevacizumab-treated group (3.23) was removed for graphing purposes, but was included in the statistical analysis. Statistical analyses panels B&C: linear mixed model.
Internalization of bevacizumab by CD133\(^+\) cells from glioblastoma into actin-containing membrane ruffles that is blocked by an inhibitor of macropinocytosis

To confirm that CD133\(^+\) cells are able to take up bevacizumab, we analyzed CD133\(^+\) cells isolated from xenograft tumors from two different patients with glioblastoma. The CD133\(^+\) cells are also SOX2\(^+\) (Figure 9). The cells were seeded on laminin in NBM without EGF and bFGF to replicate the nutrient-starved condition in glioblastoma tumors. We found that, under these conditions, bevacizumab was internalized rapidly (5 min) by the CD133\(^+\) cell isolates from both patients (Figure 10A).

Double-label immunofluorescence of the CD133\(^+\) cells for actin (Alexa-488-phalloidin; green) and bevacizumab (Alexa-647-anti-human IgG; magenta) showed that bevacizumab was internalized into membrane ruffles surrounded by actin (Figure 10B). This was suggestive of macropinocytosis, which is differentiated from other types of endocytosis by the requirement for remodeling of the actin cytoskeleton with polymerized actin in membrane ruffles. Macropinocytosis is blocked by inhibitors of the Na\(^+\)/H\(^+\) ion exchange pump, such as 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) (Commisso et al., 2013; Koivusalo et al., 2010; Lim and Gleeson, 2011), and tetramethylrhodamine(TMR)-70-kDa-Dextran is known to be internalized by macropinocytosis. (Commisso et al., 2013) The macropinocytic capabilities of the CD133\(^+\) cells were confirmed by their rapid internalization of TMR-70-kDa-Dextran (5 min) through a mechanism that was inhibited by EIPA (Figure 10C&D). The internalization of bevacizumab by the two isolates of CD133\(^+\) cells was inhibited significantly by pretreatment with EIPA (Figure 11A&B). In contrast, the internalization of bevacizumab was not inhibited significantly by EIPA
pretreatment of the paired non-stem tumor cells (Figure 11C&D). Taken together, these data suggest that CD133+ cells, but not the paired CD133-negative (non-stem) tumor cells, utilize macropinocytosis to internalize bevacizumab.
**Figure 9.** Confirmation of cancer stem-like cell marker SOX2 expression in vitro. CD133+ GBM tumor cells are SOX2+. CD133+ cells were plated onto laminin in NBM without bFGF or EGF for 18 hours (37°C, 5% CO₂), followed by the addition of bevacizumab (250 µg/ml) for 5 minutes, the cells washed, fixed, reacted with Alexa-488 anti-human IgG (5 µg/ml) (green) and with the monoclonal antibody anti-SOX2 (2.5 µg/ml), followed by an Alexa-594-secondary antibody (red), DAPI nuclear stain, cover slipping and microscopy.
Figure 10. CD133+ cells internalize bevacizumab and can internalize extracellular material into membrane ruffles in a mechanism consistent with macropinocytosis. A&B, CD133+ cells were plated onto laminin in NBM without bFGF or EGF for 18 hours (37°C, 5% CO₂), followed by the addition of bevacizumab (250 µg/ml) for 5 min, the cells washed, fixed, reacted with Alexa-647-anti-human IgG (5ug/ml) and Alexa-488-phalloidin (5 units/ml), followed by DAPI nuclear stain, cover slipping, and confocal microscopy performed. Percent of CD133+ cells from two different tumor isolates (08-387 and 4121) containing bevacizumab-positive vesicles (A). A z-stack of the double-labeling is shown (CD133+ 08-387 cells) (B). White arrows denote phalloidin-stained cell membrane (green), and red arrows denote bevacizumab-positive vesicle (magenta) surrounded by an actin-rich cytoplasmic protrusion (red).
by actin. Scale bar denotes 5µm. C&D, CD133+ cells (08-387) treated with 50 µM EIPA or vehicle for 30 minutes (37°C, 5% CO₂), followed by addition of TMR-70-kDa-Dextran (1mg/ml) (red) for 5 minutes, were washed, reacted with Alexa-488-Phalloidin (green), nuclei stained with DAPI, cover slipped and microscopy performed. In cells treated with vehicle, TMR-70-kDa-Dextran is found in membrane ruffles (white arrows), containing polymerized actin (red arrows) (D). In cells treated with EIPA, reduced amounts of TMR-70-kDa-Dextran are internalized (C&D). The area (µm²) of TMR-70-kDa-Dextran/cell is plotted as the mean±SEM from >100 cells/condition (C). Scale bars denote 10µm. Statistical analyses: exact two-sided Wilcoxon rank-sum tests.
CD133(+) Cells

A

Vehicle

EIPA

B

Area of Bev per Cell (μm^2)

Vehicle

EIPA

p=0.04

p=0.01

08-387

4121

Paired CD133(-) Tumor Cells

C

Vehicle

EIPA

D

Area of Bev per Cell (μm^2)

Vehicle

EIPA

p=0.04

p=0.01

08-387

4121
**Figure 11.** *Bevacizumab is internalized by CD133+ cells by a mechanism consistent with macropinocytosis.* CD133+ GBM cells (08-387 and 4121) were plated on laminin in NBM as in Figure 10, and paired non-stem tumor cells (CD133-negative 08-387 and 4121) were plated in DMEM with 10% FBS for 18 hours. CD133+ cells or the paired CD133-negative tumor cells were treated with vehicle or 50 µM EIPA for 30 minutes as in Figure 10C&D, followed by addition of bevacizumab (5 minutes), and the cells washed, fixed, reacted with Alexa-488-anti-human IgG, followed by DAPI nuclear stain, cover slipping and microscopy. Arrowheads denote bevacizumab-positive vesicles which are reduced in CD133+ cells treated with EIPA (A&B) but are not reduced in CD133-negative tumor cells treated with EIPA (C&D). The area (µm²) of bevacizumab/cell is plotted as the mean±SEM from >100 cells/condition (B&D). Scale bars denote 10µm. Statistical analyses: exact two-sided Wilcoxon rank-sum tests.
In CD133\(^+\) cells from glioblastoma, a fraction of bevacizumab co-localizes with a marker of a recycling compartment and a fraction with a marker of the late endosome/lysosome. Internalized receptors or proteins can be recycled to the cell surface or targeted for degradation. The routes are differentiated based on their kinetics and the markers expressed on the intracellular vesicles. Rab4 marks a ‘fast’ recycling compartment (t\(_{1/2}\) ~ 5 min) and also is involved in ‘slower’ recycling through the endosomal recycling complex (t\(_{1/2}\) ~15-30 min) (El-Sayed and Harashima, 2013; van der Sluijs et al., 1992). LAMP1 is a marker of the late endosome/lysosome. (El-Sayed and Harashima, 2013) We therefore examined the colocalization of bevacizumab with Rab4 and LAMP1. (El-Sayed and Harashima, 2013; van der Sluijs et al., 1992) CD133\(^+\) cells were seeded as described above on laminin and colocalization determined at various time points after treatment with bevacizumab. Rapid, transient colocalization with Rab4 was observed. At five minutes bevacizumab was predominantly (68%) colocalized with Rab4, with this colocalization dissipating at 30 minutes (20%) (Figure 12A&B). At three hours, a large fraction of bevacizumab was colocalized with LAMP1 (~55%) (Figure 12C&D). Similar results were observed on analysis of CD133\(^+\) cells isolated from a different glioblastoma tumor (Figure 13A&B). Analysis of human IgG by CD133\(^+\) cells using the same protocol revealed a similar pattern of trafficking (Figure 13C&D). In sections from three different tumor-bearing mice administered bevacizumab, we found co-localization of bevacizumab with Rab4 or LAMP1 in perivascular tumor cells (Figure 14A&B), suggesting similar trafficking of bevacizumab in a xenograft mouse model of glioblastoma as found in CD133\(^+\) cells \textit{in vitro}. Similar trafficking of rat IgG was found in perivascular tumor cells in the established immunocompetent model of glioblastoma treated with rat anti-mouse-VEGF-A (Figure 15A&B).
Figure 12. Co-localization of a fraction of bevacizumab with Rab4 and a fraction with LAMP1 in CD133+ cells in vitro. CD133+ GBM cells (08-387) were plated for 18 hours as in Figure 9, followed by addition of bevacizumab (250 µg/ml) for 5 minutes, the cells washed and fixed or the media replaced and the cells washed and fixed at the indicated times. The cells were reacted with Alexa-488-anti-human IgG and anti-Rab4 (1:33 dilution of lot#OL196003) or anti-LAMP1 (3.3 µg/ml) antibody, Alexa-594-conjugated secondary antibody, and DAPI nuclear stain,
followed by confocal microscopy. Arrows denote co-localization of bevacizumab (green) with Rab4 or LAMP1 (red) (A&C). The percent bevacizumab co-localized with Rab4 or LAMP1 is plotted as the mean±SEM at the indicated times based on the Mander’s coefficient (B&D).

Statistical analyses: Panels B&D, two-sided exact Wilcoxon rank-sum tests. Scale bars denotes 10µm.
**Figure 13.** Co-localization of different fractions of bevacizumab or human IgG (hIgG) with Rab4 or LAMP1 in CD133+ GBM cells in vitro. CD133+ GBM cells (4121 in A&B; and 08-387 in C&D) were plated for 18 hours as in Figure 12, followed by addition of bevacizumab (A&B) or hIgG (C&D) (250 µg/ml) for 5 minutes, the cells washed and fixed or the media replaced and the cells washed and fixed at 3 hours. The cells were reacted with Alexa-488-anti-hIgG and anti-Rab4 or anti-LAMP1 antibody, followed by Alexa-594-conjugated secondary antibody, DAPI nuclear stain, and photographed by confocal microscopy. Arrows denote co-localization of bevacizumab (green) with Rab4 or LAMP1 (red) (A&B). The percent bevacizumab or hIgG co-localized with Rab4 or LAMP1 is plotted as the mean±SEM based on the Mander’s coefficient at the indicated times.
**Figure 14.** Co-localization of a fraction of bevacizumab with Rab4 and a fraction with LAMP1 in SOX2+ perivascular tumor cells in vivo. Sections of xenograft GBM tumor from mice that were treated with bevacizumab as in Figure 2 were reacted with Alexa-488-anti-human IgG and anti-Rab4 or anti-LAMP1 antibody, and Alexa-594-conjugated-secondary antibody, followed by DAPI nuclear stain and confocal microscopy. Arrowheads or arrows denote co-localization of bevacizumab (green) with Rab4 (red) (A) or with LAMP1 (red) (B). The box in the far-left image in panels A and B is magnified in the subsequent images. Scale bar denotes 5 µm.
Figure 15. Co-localization of different fractions of rat anti-mouse VEGF-A IgG with Rab4 or LAMP1 in an established tumor in the immune competent mouse model of GBM in vivo show similar trafficking patterns. Administration of rat anti-mouse VEGF-A IgG to an established tumor in the immune competent mouse model of GBM results in highly similar trafficking of rat IgG in the perivascular tumor cells. Tumors were induced, and then once established treated with rat anti-mouse VEGF-A IgG for two weeks, followed by euthanasia, brain harvest and fixation, as described in Figure 5. Sections of tumor from four tumor-bearing mice were reacted with Alexa-488-anti-rat IgG (green), and anti-Rab4 or anti-LAMP1 antibody, and Alexa-594-conjugated secondary antibody (red), followed by DAPI nuclear stain and confocal microscopy. A fraction of the rat anti-mouse VEGF-A IgG is colocalized with Rab4 (yellow) (arrows) (A) and a fraction of the rat anti-mouse VEGF-A IgG is colocalized with LAMP1 (yellow) (arrows) (B).
Bevacizumab binds VEGF-A with high affinity and CD133<sup>+</sup> cells from glioblastoma synthesize and secrete VEGF-A. Potentially, the bevacizumab-VEGF-A complex could be trafficked differently from free bevacizumab. To characterize the trafficking of bevacizumab-VEGF complexes in CD133<sup>+</sup> cells, biotinylated-VEGF-A was incubated with bevacizumab for one hour and the mixture added to the CD133<sup>+</sup> cells for five minutes, and either fixed or fresh media added for three hours before fixing. In this experiment, the Mander’s coefficient was calculated as a measure of the percentage of bevacizumab (green) co-localized with VEGF (magenta) that was co-localized with Rab4 or LAMP1 (red) (ImageJ with JACoP-plug-In). At five minutes, 76% of the bevacizumab-VEGF complex was co-localized with Rab4 (Figure 16A&C), and at three hours, 47% was co-localized with LAMP1 (Figure 16B&D). These data suggest that in the glioblastoma CD133<sup>+</sup> cells, the unbound bevacizumab and the bevacizumab-VEGF-A complex are trafficked similarly.
Figure 16. Co-localization of a fraction of the bevacizumab-VEGF complex with Rab4 and a fraction with LAMP1 in CD133+ GBM tumor cells. CD133+ GBM cells (08-387) were plated for 18 hours as in Figure 12. Bevacizumab (250 µg/ml) was incubated with biotinylated-rec-human-VEGF (100 ng/ml) for 1 hour, and the mixture added to the cells for 5 minutes, the cells washed and fixed or the media replaced and the cells washed and fixed at 3 hours. A&B, The cells were reacted with Alexa-488-anti-human IgG and anti-Rab4 or anti-LAMP1 antibody, and Alexa-594-conjugated secondary antibody, as well as Alexa-647-Streptavidin, followed by DAPI nuclear stain and confocal microscopy. Arrows denote bevacizumab (green), Rab4 or LAMP1 (red) and VEGF (magenta), as well as their co-localization (A&B). Triple labeling is indicated by the arrows in both A & B. C&D, The percent bevacizumab-VEGF complex co-localized with Rab4 (5 minutes) (C) or LAMP1 (3 hour) (D) is plotted as the mean±SEM based on the Mander’s coefficient. Scale bars denote 5µm.
The neonatal Fc receptor (FcRn) is known to recycle human IgG in ECs. (Roopenian and Akilesh, 2007; Ward et al., 2005) We confirmed that FcRn expression was detectable in isolates of normal brain ECs (376 and 422) by western blotting using two different FcRn-specific antibodies. However, FcRn expression was not detectable in CD133+ cell isolates on western blotting using either of these antibodies (Figure 17A-C). FcRn was detectable in the isolates of the paired CD133-negative non-stem tumor cells (Figure 17A). On stripping of the membrane and re-probing for SOX2, SOX2 expression was detected in the CD133+ cells whereas a faint band representing SOX2 was detectable in one isolate of the CD133-negative non-stem tumor cells (Figure 17A). Sections from five xenograft tumors were double-labeled for FcRn and vWf, or FcRn and SOX2, and we found 72% of the FcRn fluorescence (adjusted mean) colocalized with vWf in endothelial cells and 16% of the FcRn fluorescence (adjusted mean) colocalized with SOX2+ cells (Figure 18), suggesting that the large majority of SOX2+ cells do not express FcRn in vivo. As it has been suggested that FcγRIIB can recycle immune complexes in dendritic cells (Bergtold et al., 2005), we also examined expression of FcγRIIB on the CD133+ cells. Myeloma cells (MM.1R) transduced with FcγRIIB were used as a positive control. FcγRIIB was not detected on the CD133+ cells (Figure 17B&C).
**Figure 17.** *CD133+ GBM cells do not express FcRn.* A, CD133+ cells (08-387 and 4121) were detergent lysed from cells in suspension culture in NBM without addition of EGF and bFGF; CD133-negative non-stem tumor cells cultured in DMEM with 10% FBS were washed and lysed; and primary brain ECs (isolate #422 or 623) plated on collagen in the recommended media with 10% FBS were washed and detergent lysed. Lysates (50 µg per sample) were electrophoresed on SDS-PAGE, and western blotted with the indicated antibodies (A). B&C, CD133+ GBM cells 4121 (B) or 08-387 (C), as well as the control of brain ECs (#422), were plated as above. MM.1R human myeloma cells transfected with FcγRIIB (CD32B) and plated in 10% FBS served as a positive control for expression of FcγRIIB. Detergent lysates (30 µg) of all cells were subjected to SDS-PAGE, and then western blotted with mouse anti-FcRn (Novus Biologicals #NBP2-42214), stripped and re-probed with goat anti-FcγRIIB (R&D Systems #AF1330), and then stripped and re-probed with goat anti-actin antibody (Santa Cruz #sc-1615).
Figure 18. FcRn staining largely colocalizes with the endothelial cell marker vWF in xenograft GBM tumors. A, Sections of five GBM xenograft tumors were reacted with rabbit anti-FcRn and mouse anti-vWF, or with rabbit anti-FcRn and mouse anti-SOX2 followed by Alexa-488- and Alexa-594-secondary antibodies, DAPI nuclear stain, and microscopy. B, Double-labeled cells for FcRn and vWF, or SOX2 and FcRn were counted in five fields from each tumor, and the data plotted as the mean±SEM. We found significantly more expression of FcRn in the vWF-positive cells as compared to the SOX2+ cells (adjusted mean 72.1 vs. 15.9, respectively, p<0.0001). Statistical analysis: linear mixed model.
Effects of bevacizumab on the expression of VEGF-A&C and PGF by CD133+ cells

Bevacizumab neutralizes VEGF-A and, thus, could induce VEGF-A-depletion in treated cells. VEGF-C also binds VEGFR2 and activates its signaling. (Joukov et al., 1996) Bevacizumab can induce expression of placental growth factor (PGF) (a VEGF family member), which binds and activates VEGFR1 and may indirectly activate VEGFR2 through several mechanisms. (De Falco, 2012; Simon et al., 2014) Therefore, we examined the expression of VEGF-A, VEGF-C, and PGF in the CD133+ cells plated as above (in the absence of added growth factors) and treated with bevacizumab. VEGF-A, VEGF-C and PGF were detected in the media of CD133+ cells, and the levels increased from 24 to 48 hours (Figure 19A-C). With bevacizumab treatment, the levels of VEGF-A were markedly reduced whereas the levels of VEGF-C and PGF were unchanged (Figure 19A-C). Although there was a reduction in phospho-VEGFR2 in the CD133+ cells on bevacizumab depletion of VEGF-A, phospho-VEGFR2 was still detectable (Figure 19D&D).
Figure 19. Bevacizumab decreases secreted VEGF-A in CD133+ cells but has no effect on VEGF-C or PGF, and does not eliminate VEGFR2 activation. A-C, CD133+ cells were plated as in Figure 9 in NBM without addition of EGF and bFGF, treated with bevacizumab or hlgG for 24 or 48 hours (1 mg/ml), and the media removed for ELISA assay (A-C). Levels of VEGF-A or VEGF-C or PGF protein in the media of CD133+ cells treated with bevacizumab or vehicle are graphed as the mean±SEM at the time points shown. The levels of VEGF-A with bevacizumab treatment at 24 and 48 h were 9.18±0.32 pg/ml and 8.04±0.34 pg/ml (mean±SEM), respectively. D&E, CD133+ GBM cells were plated and treated with bevacizumab as described in Figure 9 for 48 hours. Cells were then reacted with a mouse monoclonal antibody anti-VEGFR2 and rabbit anti-phospho-tyrosine antibodies, followed by Alexa-488- and Alexa-594-secondary antibodies, DAPI nuclear stain, and microscopy (D). Quantitation of colocalization from 10 fields/condition was performed using ImageJ, and the colocalization plotted as the mean±SEM (E). Statistical
analyses: Panels A-D, two-sided exact Wilcoxon rank-sum tests. *Denotes p value < 0.05; **denotes p value < 0.01; ***denotes p value < 0.001; and ****denotes p value < 0.0001. n.s., denotes not significant.
Macropinocytosis of bevacizumab promotes proliferation of CD133\(^+\) cells and bevacizumab depletion of VEGF-A induces a pro-survival autophagy

To determine whether bevacizumab depletion of VEGF-A induced autophagy in the CD133\(^+\) cells, we examined the colocalization of LC3 puncta with the lysosomal marker LAMP2 (Galluzzi et al., 2015), as well as examining the effect of bevacizumab on cell cytotoxicity/death and viability/proliferation. The concentration of bevacizumab we used had been reported previously to induce autophagy in glioblastoma and cancer stem cell-like cells. (Liang et al., 2015) We found significantly higher autophagy and no change in cell death in bevacizumab-treated CD133\(^+\) cells as compared to vehicle at 24 hours (Figure 20A&B). Treatment with hIgG at the same concentration decreased autophagy and had no effect on cell death at 24 hours (Figure 20A&B). Downregulation of VEGF-A with siRNA in the CD133\(^+\) cells increased the colocalization of LC3 puncta with LAMP2 as compared to the control siRNA at 48 hours (Figure 21), suggesting bevacizumab depletion of VEGF-A in the media causes a growth factor-starvation-induced autophagy. To optimally assess viability/proliferation, an MTT assay was performed at 48 hours, and indicated that bevacizumab treatment resulted in higher viability/proliferation as compared to the vehicle control (Figure 20C). These results suggest macropinocytosis of human IgG, followed by its lysosomal trafficking and non-specific degradation provided basic building blocks that promoted viability/proliferation. Blocking macropinocytosis of bevacizumab with EIPA treatment at 48 hours significantly increased autophagy and cell death (Figure 20D&E). EIPA treatment also increased autophagy and cell death in vehicle-treated CD133\(^+\) cells, but the increase in cell death was greater in the absence of bevacizumab treatment (Figure 20D). Treatment with BafilomycinA1, which blocks lysosomal
acidification and autophagy (Galluzzi et al., 2015), caused a significant reduction in co-localization of LC3 puncta with LAMP2 in the bevacizumab-treated CD133⁺ cells and an increase in cell death (Figure 20D&E). Taken together, these data suggest that macropinocytosis and lysosomal trafficking of bevacizumab and other proteins promotes CD133⁺ cell survival and proliferation in vitro, and that autophagy induced by bevacizumab depletion of VEGF-A also promotes survival.
Figure 20. Bevacizumab trafficking in CD133+ cells affects two survival pathways. CD133+ cells were plated as in Figure 9 in NBM without addition of EGF and bFGF, treated with bevacizumab or hlgG for 24 or 48 hours (1 mg/ml), and the cells washed, fixed and reacted with anti-LC3 antibody (2 μg/ml) (green) and anti-LAMP2 antibody (4 μg/ml) (red), followed by Alexa-488 and
Alexa-594-conjugated secondary antibodies, DAPI nuclear stain and confocal microscopy (A, D&E), or the cells subjected to a cytotoxicity/death assay (B&D) or an MTT assay (C). A, Colocalization of LC3-LAMP2 puncta/cell in CD133+ cells treated with bevacizumab, hlgG or vehicle for 24 hours. Quantitation of the area of colocalization (ImageJ) from ten fields/condition is graphed as the mean±SEM. B, Quantitation of cell cytotoxicity/death (relative to vehicle) after bevacizumab, hlgG or vehicle treatment for 24 hours, and graphed as the mean±SEM. C, Quantitation of viability/proliferation in CD133+ cells treated with bevacizumab or vehicle for 48 hours. D&E, Colocalization (ImageJ) of LC3 puncta with LAMP2 (10 fields/condition) or quantitation of cell cytotoxicity/death (relative to vehicle) is shown in CD133+ cells treated with bevacizumab or vehicle for 48 hours, with or without inhibitors, and is graphed as the mean±SEM (D). Representative images of the colocalization of the LC3-LAMP2 puncta in the same six conditions is shown (H). Arrows denote co-localization (yellow) (E).

Statistical analyses: Panel A and B, the Steel Method; C, two-sided exact Wilcoxon rank-sum test; and D, Wilcoxon rank-sum tests. *Denotes p value < 0.05; **denotes p value < 0.01; ***denotes p value < 0.001; and ****denotes p value <0.0001. n.s., denotes not significant.
Figure 21. *Downregulation of VEGF-A induces autophagy in CD133+ GBM cells.* Downregulation of VEGF-A with siRNA induces colocalization of LC3-LAMP2 puncta. CD133+ cells were treated with siVEGF-A or control siRNA for 48 hours. Cells were then reacted with anti-LC3 antibody (2 µg/ml) and anti-LAMP2 antibody (4 µg/ml), followed by Alexa-488- and Alexa-594-secondary antibodies, DAPI nuclear stain, and confocal microscopy. Quantitation of the number of LC3-LAMP2 puncta/cell (ImageJ) is graphed as the mean±SEM. Statistical analysis: Wilcoxon rank-sum test (p=0.04).
CHAPTER
4

DISCUSSION

We demonstrate that bevacizumab gains access to the perivascular tumor niche in established orthotopic mouse models of glioblastoma through the well-described alterations in the blood-brain barrier, suggesting that vascular normalization by bevacizumab does not occur in 100% of tumor vessels. We found that the CD133⁺/SOX2⁺ cells and the paired non-stem tumor cells can internalize bevacizumab but do so through different mechanisms and that, in vivo, SOX2⁺/CD44⁺ perivascular tumor cells appear to largely be responsible for anti-VEGF-A IgG internalization. We further show that the intracellular trafficking of anti-VEGF-A IgG in glioblastoma tumors affects the survival of CD133⁺/SOX2⁺ cells.
Under the conditions used in these experiments, the CD133+ cells internalized bevacizumab and hIgG through the process of macropinocytosis, based on our observation of actin-enriched plasma membrane ruffles surrounding and engulfing bevacizumab into large vesicles and significant inhibition of internalization by EIPA. While macropinocytosis appeared to be the primary mechanism underlying the internalization of bevacizumab in the CD133+ cells, we cannot rule out the possibility of a minor role for another clathrin-independent mechanism. The paired non-stem tumor cells internalized much less bevacizumab and, as the internalization was not affected significantly by treatment with EIPA, macropinocytosis did not appear to contribute to the internalization in these cells.

Analysis of the intracellular trafficking of bevacizumab and hIgG in vitro showed colocalization with established markers of endocytic compartments in CD133+ cells, providing clues to the potential fate of the internalized bevacizumab. Under the experimental conditions without added growth factors, bevacizumab was largely co-localized with a marker of a ‘fast’ recycling compartment (Rab4) at five minutes. This would suggest that a considerable amount of the internalized bevacizumab is recycled rapidly to the extracellular environment where it would be available to bind and neutralize VEGF-A. FcRn has been shown to be responsible for the recycling of endogenous IgG in ECs and several other cell types (reviewed in (Roopenian and Akilesh, 2007)). The time course for bevacizumab and human IgG recycling by the CD133+ cells was faster than has been described for FcRn. (Ward et al., 2005) Moreover, we found that the CD133+/SOX2+ cells do not express FcRn by western blot analysis and that the large majority of SOX2+ perivascular tumor cells do not express FcRn in vivo. Thus, FcRn was unlikely to play a role in the recycling of bevacizumab and hIgG in the CD133+ cells. Similarly,
the inhibitory FcγRIIB has been shown to endocytose and recycle immune complexes back to the cell surface on dendritic cells (Bergtold et al., 2005), but we did not detect FcγRIIB on the CD133^+ cells by western blotting. The isoelectric point (pI) of humanized IgG mAbs has been shown to affect clearance with a higher pI promoting clearance in a manner that was FcRn independent and presumed to be due to an increased rate of fluid phase endocytosis (reviewed in (Gurbaxani et al., 2013)). Bevacizumab has a higher pI (pI=8.3) (http://www.drugbank.ca) than endogenous IgG (pI=7.2). Although we did not compare the rates of internalization of bevacizumab and hIgG, we did find similar trafficking patterns in the CD133^+ cells.

Delivery of the endosome cargo to the late endosome/lysosome is slower than its delivery to the recycling compartment. (El-Sayed and Harashima, 2013) We found that in the CD133^+ cells a large amount of bevacizumab colocalized with a marker of the late endosome/lysosome (LAMP1) at three hours. This is consistent with the report of localization of bevacizumab to the lysosome in retinal ECs at 72 hours post-treatment in vitro. (Deissler et al., 2012) This localization to the lysosome suggests that the CD133^+ cells in the perivascular tumor space also degrade bevacizumab. The percentage of bevacizumab localized to the LAMP1 compartment is probably an underestimate as that pool of bevacizumab is undergoing degradation. In the early endosome, differential sorting of cargo protein to a recycling compartment or the lysosome can be based on specific cargo protein signals (El-Sayed and Harashima, 2013), including ubiquitination that signals sorting of membrane proteins to the lysosome. (Mohapatra et al., 2013)
The trafficking of the bevacizumab-VEGF-A complex was similar to the trafficking of bevacizumab alone in the CD133+ cells; a fraction co-localized with a marker of the ‘fast’ recycling compartment (Rab4) at five minutes (75%) and a fraction co-localized with a marker of the late endosome/lysosome (LAMP1) (47%) at three hours. In ECs, the VEGF-VEGFR2 complex is known to be internalized and recycled by different mechanisms, including clathrin-coated pit and endophilin-dependent mechanisms (Boucrot et al., 2015; Lampugnani et al., 2006), but in tumor cells the internalization and trafficking of the VEGF-VEGFR2 complex is not as well-defined.

We show that bevacizumab trafficking directly affects two survival pathways in the CD133+ cells. First, bevacizumab in the perivascular space depletes CD133+ cell-secreted VEGF-A causing a growth factor-deprivation-induced autophagy. This is demonstrated by the significantly higher number and area of LC3-LAMP2 puncta when the CD133+ cells are treated with bevacizumab as compared to vehicle; an increase in numbers of LC3-LAMP2 puncta when VEGF-A is downregulated with siRNA but not when the cells are treated with control siRNA; and a decrease in LC3-LAMP2 puncta on treatment of the cells with hlgG. BafilomycinA1 treatment (inhibits lysosomal acidification) blocked the increase in number and area of LC3-LAMP2 puncta in CD133+ cells treated with bevacizumab. The significant decrease in cell death with bevacizumab treatment at 48 hours supports the concept that bevacizumab induces a pro-survival form of autophagy in the CD133+ cells. Autophagy is generally a survival mechanism by which cells that are stressed due to nutrient deprivation, absence of growth factors, or hypoxia metabolize organelles, misfolded proteins and aggregated proteins, thereby generating molecules that allow the cell to survive. (Galluzzi et al., 2015; Liu et al., 2013) Previously, others have
reported an increase in autophagy on bevacizumab treatment of glioblastoma cells cultured in media with growth factors or under hypoxic conditions (Hu et al., 2012; Liang et al., 2015), but the effect of bevacizumab on VEGF-A secreted by tumor cells was not examined. Secondly, our data suggest that the macropinocytosis of bevacizumab followed by its targeting to the lysosome promotes the survival of CD133$^+$ cells due to the increased availability of basic building blocks (amino acids) for the cell. This is supported by the increased viability/proliferation we observed with bevacizumab treatment, and the significant increase in cell death we observed on inhibition of macropinocytosis using the inhibitor EIPA. EIPA treatment also significantly increased autophagy presumably by blocking the macropinocytosis of nutrients and bevacizumab by the cell. The increase in cell death and autophagy taken together point to the possibility that EIPA induces autophagic cell death. The increase in cell death associated with EIPA treatment was significantly lower in bevacizumab-treated CD133$^+$ cells than vehicle-treated CD133$^+$ cells, and this is likely due to the protective growth factor-deprivation-induced autophagy that bevacizumab promotes in the CD133$^+$ cells.

Macropinocytosis is highly regulated and thought to be stimulated by growth factors. (El-Sayed and Harashima, 2013; Lim and Gleeson, 2011) The macropinocytosis of bevacizumab by CD133$^+$ cells occurred in the absence of exogenous growth factors, suggesting that an autocrine or paracrine growth factor pathway may be promoting this process, such as the VEGF/VEGFR2 pathway or another growth factor signaling pathway. VEGF-A has been reported to be a pro-angiogenic growth factor secreted by CD133$^+$ cells from glioblastoma. (Bao et al., 2006b) Our data support this concept and further suggest that the CD133$^+$ cells are addicted to VEGF-A. When VEGF-A is depleted by bevacizumab treatment the cells undergo autophagy. Despite
depletion of VEGF-A with bevacizumab, phosphorylated-VEGFR2 was still detectable in CD133+ cells although the level of phosphorylation was reduced. This could be due to CD133+ cell secretion of VEGF-C, and/or to a ligand-independent VEGFR2 signaling mechanism. Growth factor receptors also can be macropinocytosed by cells, e.g., the macropinocytosis and lysosomal targeting of EGFR when bound by a combination of mAb anti-EGFR (cetuximab) and anti-human IgG has been reported in large vessel endothelial cells transfected with EGFR. (Berger et al., 2012)

Our studies of the trafficking of anti-VEGF antibody when administered to established xenograft and syngeneic mouse models of glioblastoma indicate the IgG gains access to the perivascular tumor tissue and is internalized predominantly by perivascular SOX2+/CD44+ -tumor cells. Furthermore, in these cells, a fraction of bevacizumab is co-localized with Rab4 and a fraction with LAMP1, suggesting similar trafficking of bevacizumab in vivo as we found in CD133+/SOX2+ cells in vitro. Supporting our in vitro data, in bevacizumab-treated xenograft tumors there was increased colocalization of LC3 puncta with LAMP2 in perivascular tumor cells as compared to placebo-treated tumors, suggesting bevacizumab induced autophagy in vivo in the xenograft model. Other investigators have shown that bevacizumab treatment induces autophagy in tumor cells in glioblastoma xenograft models and this was attributed to upregulation of interferon regulatory factor-1 or hypoxia in tumor cells. (Hu et al., 2012; Liang et al., 2015)

In summary (Figure 22), our data suggest that (i) CD133+/SOX2+ cells in media without addition of growth factors macropinocytose bevacizumab, and a fraction is trafficked to a ‘fast’ recycling
compartment independent of FcRn and a fraction to the lysosome; (ii) Bevacizumab trafficking promotes CD133+ cell survival through two pathways: the macropinocytosis and lysosomal trafficking of bevacizumab that generates basic building blocks for the cell and growth factor deprivation-induced autophagy; and (iii) In vivo, anti-VEGF-A antibody localizes to the perivascular tumor tissue suggesting it can target tumor-secreted VEGF, is similarly trafficked in perivascular tumor cells and induces a pro-survival autophagy. It should be noted that we are not suggesting bulk effects on the levels of bevacizumab. Rather, we demonstrate a significant effect on the survival of a population of tumor cells in the perivascular microenvironment that are resistant to radiation and chemotherapy and have been suggested to be cancer stem cell-like cells. We have not addressed whether the uptake of bevacizumab could be affecting other clinically relevant processes, such as the expression of the glucose transporter GLUT3 (Kuang et al., 2017), the consequences of the survival of the perivascular cancer stem-like cells after bevacizumab treatment, or the mechanisms that trigger their subsequent expansion and potential aggressive invasiveness. (Lu-Emerson et al., 2015) As bevacizumab also is used to treat renal, colon and lung cancer and as we observed similar trafficking of bevacizumab and hIgG, these studies may have applicability to other cancers in which FcRn is not expressed on cancer stem cell-like cells or tumor cells and to other mAb therapeutics.
Figure 22. Schematic depicting the effect of bevacizumab trafficking on two survival pathways in CD133+ cells from glioblastoma. Bevacizumab gains access to the perivascular tumor tissue by leakage across an altered BBB, followed by macropinocytosis by CD133+ cells and trafficking to a Rab4 recycling compartment (not shown) or a LAMP1 lysosomal compartment, as well as bevacizumab neutralization of VEGF-A in the perivascular space inducing autophagy.

Macropinocytosis of bevacizumab results in partial trafficking to the lysosome where non-specific degradation occurs, and this generates amino acids (basic building blocks) for the cell that promotes survival/proliferation. The depletion of VEGF in the extracellular environment by bevacizumab results in growth factor starvation-induced autophagy that also promotes survival.
Bevacizumab therapy for recurrent glioblastoma is not effective for all patients with 20% not responding. A method to identify if a patient will respond to bevacizumab therapy prior to the start of treatment would prevent an ineffective and expensive treatment and allow the patient the opportunity to participate in clinical trials of other drugs that could potentially show efficacy. A DNA fingerprint – a signature of combined gene amplifications, deletions, and somatic mutations – could be designed based on copy number and exome sequence variations between responders and non-responders to bevacizumab therapy.

Copy number variation (CNV) is the analysis of DNA amplification and deletion, which is different from RNA-based tests that determine the state of the cell at any given moment. The advantage of studying the DNA status of a patient is that the alterations in DNA change more slowly over time as compared to transcriptional RNA levels.

Preliminary analysis of gene copy number variation in DNA from 18 responders and 16 non-responders to bevacizumab treatment has identified more than 100 candidate genes that are differentially amplified or deleted between the two groups using the PennCNV-tumor algorithm. (Wang et al., 2007) For this pilot study, DNA subjected to the Affymetrix SNP 6.0 DNA array was extracted from formalin-fixed paraffin-embedded (FFPE) tumor biopsy sections from adult patients with recurrent glioblastoma who were identified and grouped based on the following clinical definition determined prior to beginning the study: Responders had stable disease at 16 weeks post-initiation of bevacizumab therapy whereas non-responders had tumor progression
within 8 weeks using the Revised Assessment in Neuro-Oncology (RANO) criteria. (Wen et al., 2010) The DNA array results included a higher occurrence of PTEN deletion in the non-responders consistent with intact PTEN correlating with better response and this was found in a prior clinical trial, confirming the validity of the results. (D’Alessandris et al., 2013) The DNA array results identified genes involved in cellular pathways commonly altered in tumor cells including a set of altered genes involved in endocytosis and trafficking (data not shown).

Mining the TCGA database, we found a significant incidence of amplification or heterozygous deletion in five candidate genes in glioblastoma (data not shown). These five genes play critical roles in the trafficking of molecules and cell function. The level of expression of proteins that function in the endocytic pathways is thought to be critical, as over-expression or down-regulation of multiple proteins in these pathways has been shown to alter protein function resulting in aberrant endocytosis or trafficking. (Doherty and McMahon, 2009; El-Sayed and Harashima, 2013; Jones et al., 2003; Sheff, 2004) This suggests amplification or heterozygous deletion of these 5 candidate genes could have deleterious effects on endocytosis and trafficking.

We will perform the DNA array and whole exome sequencing analysis to analyze CNV and sequence variants. Copy number variations can be confirmed on the whole exome sequencing data using the GISTIC2 method. (Mermel et al., 2011) GISTIC2 is used to further threshold estimated values to -2, -1, 0, 1, 2 and genes mapped onto human genome coordinates (UCSC cgData HUGO probeMap).
We will validate the relevance of our findings by data mining from public databases to determine if a similar overall incidence occurs in a larger population of glioblastoma tumors and to identify differences that are associated with endocytosis and trafficking pathways. The differential amplification or deletion of candidate genes will be confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR green dye and validated primers. In addition, immunohistochemical staining for the candidate genes can be performed using remaining FFPE tumor sections that were used for the array starting material. Sanger sequencing of PCR-amplified DNA regions containing the candidate mutations will confirm the exome sequence variants. For somatic mutations, the sequence variant must be absent from the paired white blood cell pellet.

Our goal is to create a DNA fingerprint – a genetic profile of specific gene amplifications, deletions, and mutations – to identify if a patient will respond to bevacizumab therapy. The DNA fingerprint will be composed of a panel of confirmed candidate gene alterations determined from our copy number variation and exome sequence variant analyses. The DNA fingerprint will be blindly tested on a group of patients to determine the accuracy with which the fingerprint can identify if a patient is a responder or non-responder to bevacizumab. A multivariable logistic regression statistical model will be used to determine the accuracy of the DNA fingerprint.

We anticipate that multiple candidate copy number and exome sequence variants will be identified and certain combinations may predict whether a patient will respond to bevacizumab therapy. Based on variants identified, we aim to design a DNA fingerprint that determines if a
patient with recurrent glioblastoma will not respond to bevacizumab treatment with a focus on altered genes that are part of the endocytosis and trafficking pathway. We realize that genes not involved in endocytosis may contribute significantly to the accuracy of the DNA fingerprint. There is also the possibility that the DNA fingerprint may identify response within only a specific molecular subtype of glioblastoma, so the subtype will be determined for the cases used for these analyses.

DNA extracted from FFPE sections can be degraded to a greater extent than permitted for accurate sequencing. To minimize the degradation of the DNA, FFPE sections will be stored in the freezer and must pass quality control tests that are performed during sample preparation. There are multiple analysis methods available to analyze array and sequencing data. (Bao et al., 2014; Hintzsche et al., 2016) To ensure that the proper algorithm is performed, an expert in bioinformatics will analyze the raw data and assist with the interpretation of the results.

Several studies could further delineate the importance of therapeutic monoclonal antibody trafficking in cancer stem-like cells as a potential resistance mechanism. Expanding our fingerprint to consider alterations in the DNA or RNA of all genes not enriched for certain pathways, as well as additional patient characteristics, could increase the accuracy of a DNA fingerprint for predicting whether a patient will respond to bevacizumab therapy.

As additional autophagy inhibitors become available, we will determine whether a combination of bevacizumab and such an inhibitor are more efficacious at inhibiting tumor growth in vivo and preventing pro-survival autophagy in mouse models of glioblastoma. Finally, research that
expands our current findings with bevacizumab to other monoclonal antibodies, such as ABT-806 (anti-EGFRvIII), can confirm the wider implications of the effects of cancer stem-like cell macropinocytosis and trafficking of monoclonal antibody therapeutics on efficacy and on cancer stem-like cell survival.
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relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17, 98–110.


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>BTG</td>
<td>Brain Tumor Group (of the EORTC)</td>
</tr>
<tr>
<td>CD44</td>
<td>Cell-Surface Glycoprotein 44</td>
</tr>
<tr>
<td>CD133</td>
<td>Cell-Surface Glycoprotein 133, also known as Prominin 1</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variation</td>
</tr>
<tr>
<td>CSLC</td>
<td>Cancer Stem-like Cells</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony Stimulating Factor-1</td>
</tr>
<tr>
<td>CXCL2</td>
<td>Chemokine (C-X-C motif) Ligand 2</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) Ligand 12 (also known as SDF1)</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Chemokine (C-X-C motif) Receptor 2</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosome Antigen 1</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EGFRvIII</td>
<td>Epidermal Growth Factor Receptor variant III</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-Ethyl-N-isopropyl)amiloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EORTC</td>
<td>European Organization for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal–Regulated Kinases</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc Receptor</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-Fixed Paraffin-Embedded</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast Growth Factor 2</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial Cell-Derived Neurotrophic Factor</td>
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<tr>
<td>GLUT3</td>
<td>Glucose Transporter Type 3, also known as SLC2A3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factors</td>
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<tr>
<td>hIgG</td>
<td>Humanized IgG</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>HUGO</td>
<td>Human Genome Organisation</td>
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<tr>
<td>Iba1</td>
<td>Ionized Calcium-Binding Adapter Molecule 1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal (injection)</td>
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<tr>
<td>KDR</td>
<td>Kinase Insert Domain Receptor (also known as VEGFR2)</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>KLH</td>
<td>Keyhole Limpet Hemocyanin</td>
</tr>
<tr>
<td>LAMP1</td>
<td>Lysosomal Associated Membrane Protein 1</td>
</tr>
<tr>
<td>LAMP2</td>
<td>Lysosomal Associated Membrane Protein 2</td>
</tr>
<tr>
<td>LRP-1</td>
<td>Low Density Lipoprotein Receptor-Related Protein 1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6-methylguanine-DNA Methyltransferase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility</td>
</tr>
<tr>
<td>MITC</td>
<td>5-(3-methyl-1-triazeno)imidazole-4-carboxamide</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NBM</td>
<td>Neurobasal A Media</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Nonobese diabetic-severe combined immunodeficiency (mice)</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature (compound)</td>
</tr>
<tr>
<td>OS</td>
<td>Overall Survival</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PDX</td>
<td>Patient-Derived Xenograft</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PFS</td>
<td>Progression-Free Survival</td>
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<tr>
<td>PFS-6</td>
<td>Progression-Free Survival at 6 months</td>
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<tr>
<td>PGE-2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PGF</td>
<td>Placental Growth Factor</td>
</tr>
</tbody>
</table>
pI  Isoelectric Point
PKB  Protein Kinase B
PTEN  Phosphatase and Tensin Homolog
Rab4  Ras-related Protein Rab-4A
Rab11  Ras-related Protein Rab-11
RANO  Revised Assessment in Neuro-Oncology (criteria)
RNA  Ribonucleic Acid
SDF1  Stromal-Derived Factor-1 (also known as CXCL12)
SNP  Single Nucleotide Polymorphisms
SOX2  Sex Determining Region Y-box 2 (SRY)
STR  Short Tandem Repeat
TCGA  The Cancer Genome Atlas
TEC  Tumor-associated EC
TGF-β  Transforming Growth Factor Beta
Tie  Tyrosine Kinase with Immunoglobulin-like and EGF-like Domains
TMB  3,3′,5,5′-Tetramethylbenzidine
TMR  Tetramethylrhodamine
TNFα  Tumor Necrosis Factor Alpha
TNFR1  Tumor Necrosis Factor Receptor 1
VEGF  Vascular Endothelial Growth Factor
VEGFR  Vascular Endothelial Growth Factor Receptor
vWf  von Willebrand Factor
ZO  Zonula Occludens