TARGETING STEM CELL PATHWAYS IN GLIOBLASTOMA

by

ALVARO GONZALO ALVARADO CABELLOS

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Dissertation Advisor: Dr. Justin D. Lathia

Department of Molecular Medicine

CASE WESTERN RESERVE UNIVERSITY

May, 2016
CASE WESTERN RESERVE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

We hereby approve the dissertation of

Alvaro Gonzalo Alvarado Cabellos

candidate for the degree of Doctor of Philosophy *.

Committee Chair
William Schiemann Ph.D.

Committee Member
Justin Lathia Ph.D.

Committee Member
Michael Vogelbaum MD, Ph.D.

Committee Member
Tom Hamilton Ph.D.

Committee Member
Eain Murphy Ph. D.

* We also certify that written approval has been obtained
for any proprietary material contained therein.
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Acknowledgements

I would first like to thank my mentor, Dr. Justin D. Lathia, for his continue support and guidance in all the projects presented in this thesis. We both took a chance three and a half years ago and I could not have asked for a better mentor; even with Monday meetings at 8 am. I would also like to thank all the past and current members of the Lathia Laboratory for their constructive comments and technical assistance. Their encouragement and willingness to collaborate was critical for the success of my projects.

Similarly, I would like to thank my committee members for always providing positive feedback and making me see the bigger picture. My gratitude also goes out to the Molecular Medicine PhD program and Case Western Reserve University for allowing me to experience science in a first class environment. To my fellow MolMedders (past and present), it’s been a pleasure growing with you and watching you grow. A special thanks to Jessica E. Sacks for her loving support and constant enthusiasm, when mine was lacking.

Finally, I would like to thank my family and friends in Peru for believing in me and sending me the best vibes from afar. It was not easy leaving but your love and support never left me.
Cancer stem cells (CSCs) provide an additional layer of complexity for tumor models and targets for therapeutic development. The balance between CSC self-renewal and differentiation is driven by niche components, including adhesion, soluble factors, and tumor infiltrating immune cells. Using patient-derived glioblastoma CSCs, I revealed two different signaling pathways that promote activation of self-renewal programs. While studies have demonstrated that the reduction of adhesion decreased CSCs maintenance, the molecular circuitry underlying these interactions has yet to be resolved. My results link CSC-specific junctional adhesion molecule A (JAM-A) to a microRNA regulatory network, miR-145, that is altered in glioblastoma and can be targeted to attenuate CSC self-renewal. Similarly, toll-like receptor (TLR) activation limits proliferation in developing neural systems, yet these pathways are generally drivers of proliferation in many tumors including glioblastoma. This functional shift is surprising, considering the number of developmental programs activated in CSCs. Our findings reveal a unique pathway through which TLR4 can potentially suppress the stem cell state and found to be downregulated in CSCs, linking innate immune signaling responses to stem cell maintenance. Altogether, the work presented in this dissertation describes dysregulated pathways in glioblastoma, which can drive its malignancy and tumor initiation capacity.
Chapter 1: Introduction
1.1 Introduction

The work presented in this dissertation describes the importance of stem cell pathways for tumor growth and malignancy in glioblastoma (GBM). The description of a cell population in advanced cancers capable of self-renewal and tumor progression, termed cancer stem cells (CSCs), was a major discovery in the cancer field in the last two decades (Bonnet and Dick, 1997; Galli et al., 2004; Ignatova et al., 2002; O'Brien et al., 2007; Piccinini and Midwood, 2010; Singh et al., 2003). The ability of these cells to activate and utilize stem cell pathways by different mechanisms will be explored in detail. This dissertation will first cover the concept of CSCs and describe some of the components regulating their activity that are pertinent to the discussion in subsequent sections. Next, two distinct signaling pathways, cellular adhesion and innate immunity, will be explored in detail in order to elucidate mechanisms by which cells have enhanced self-renewal and tumorigenic potential. Finally, future perspectives of CSCs and GBM will be explored based on the findings described here and the current state of anti-tumorigenic therapies.

1.2 GBM

GBM, a World Health Organization (WHO) grade IV astrocytoma, is the most common primary malignant brain tumor in adults (Ostrom et al., 2015). Primary brain tumors consist of a diverse group of neoplasms that arise from different cell lineages present in the brain (Huse and Holland, 2010). In this context, gliomas
Figure 1.1. Cells that putatively originate primary brain tumors. Self-renewing, common progenitors are thought to produce committed neuronal and glial progenitors that eventually differentiate into mature neurons, astrocytes and oligodendrocytes. Although the precise cells of origin for diffuse glioma variants and medulloblastoma remain largely unknown, a selection of likely candidates for each (dashed arrows) is indicated. (Taken from Huse, J.T. and Holland, E.C., Nature Reviews Cancer, 2010).
are derived from mature glia or their less differentiated precursors and infiltrate the surrounding brain tissue (Figure 1.1). The WHO classifies this broad group into astrocytic, oligodendroglial, and mixed (oligoastrocytic) groups (Louis et al., 2007). In addition to morphological features that resemble the cell of origin, the WHO grading system takes into account the presence of histological features that characterize a tumor into a higher grade. In the case of Astrocytomas, grade I (pilocytic astrocytoma) is considered benign while grades II (diffuse astrocytoma), III (anaplastic astrocytoma), and IV (glioblastoma) are classified as malignant. All three malignant groups present increased cellularity as well as nuclear atypia while grades II and IV are also marked by the presence of mitoses (Louis et al., 2014). Additionally, GBM can also present microvascular (endothelial) proliferation as well as pseudopalisading necrosis (Figure 1.2). Thus, GBM is generally characterized by molecular heterogeneity and very aggressive behavior based on constant mitotic activity as well as the associated increase in endothelial cell proliferation as observed histologically. GBM is a uniformly fatal malignancy with a 12-14 (Stupp et al., 2005; Wen and Kesari, 2008) median survival and a 5-year survival of less than 10% (Stupp et al., 2009). The standard of care is comprised of maximal surgical resection followed by chemotherapy and/or radiation (Stupp et al., 2009; Walker et al., 1980). Despite this aggressive treatment, clinical success is minimal and the transient effect of these therapies is eventually overcome leading to tumor recurrence in most GBM patients with a 5-year recurrence-free survival rate of less than 5% (Stupp et al., 2009). These dismal figures underscore the necessity of a better
Figure 1.2. Histopathologic features of pseudopalisades in glioblastoma (GBM). A, narrow pseudopalisades, especially those <100 um wide, have hypercellular zones surrounding internal fibrillarity but usually lack central necrosis. B, medium-sized pseudopalisades (200–400 um) are characterized by central necrosis, central vacuolization, and individual dying cells but typically have a peripheral zone of fibrillarity immediately inside the pseudopalisade. Note the absence of central vessels or vascular thrombosis in A and B. C, the largest pseudopalisades (those >500 um) have extensive necrotic zones and nearly always have central vessels. Note the distance of vessels to the inner aspect of the pseudopalisade and the numerous outpouchings of pseudopalisades (C, arrow), which on tangential sectioning could give rise to smaller pseudopalisades resembling A or B. D, GBM cells “caught in the act” of forming a pseudopalisade (arrowhead) appear to be migrating away from an enlarged, distorted, and presumably dysfunctional vessel (arrow). Note the perivascular fibrillarity, lack of central necrosis, and streaming of tumor cells along fibrillar processes in this instance. (Taken from Brat, D.J. et al., Cancer Research, 2004).
understanding of the disease at both the cellular and molecular level. In this context, there have been recent advances in trying to characterize GBM molecularly that led to a classification based on copy number variations, expression profiles, and survival. These efforts were led by The Cancer Genome Atlas (TCGA), a collaboration between the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI). In the span of 10 years it has helped understand the molecular basis of different types of cancer through the application of genome analysis, including large-scale genome sequencing. Namely, GBM tumors can be divided into 4 subgroups: classical, mesenchymal, neural, and proneural groups (Verhaak et al., 2010). From this study, patients in the proneural group have generally a better prognosis compared with patients in other groups. Similarly, patients in the classical group are the ones who respond the best to aggressive therapy while there is no significant benefit in the other groups. However, a similar study analyzing the promoter DNA methylation alterations identified a distinct glioma-CpG island methylator phenotype (G-CIMP) (Noushmehr et al., 2010). Patients from this group have a significantly better outcome compared with the other groups account for the improved survival seen in the original proneural subtype. Although these classifications have shed some light on the diverse nature of the disease and help with the development of treatment, there are still many questions that require further investigation. Especially in the light of additional intratumoral complexity found in GBM at the single cell level where samples (taken from different locations) from the same patient had characteristics of more than one molecular subtype (Patel et al.,
In this context, my work explores the cellular component of GBM in various angles. First, by studying the different cellular populations composing the tumor, second, by analyzing the communication between cells from the same population and between cells of different populations, and third, by understanding mechanisms by which these populations are able to proliferate and promote tumor formation despite therapies aimed at eradicating them. The following sections provide an outline of the different components that contribute to the complexity of malignant tumor such as GBM.

1.3 Cancer stem cells

The lack of clinical therapeutic success observed with the current standard of care can be attributed to the cellular heterogeneity, considered a hallmark of cancer (Hanahan and Weinberg, 2011), inherent to tumors. The existence of several cellular populations including endothelial, immune infiltrative, normal brain, and tumor cells adds an extra layer of complexity when trying to design therapies against GBM. Similarly, within the neoplastic cells, recent reports have described a subpopulation of tumor-driving cells capable of self-renewal in several oncogenic contexts (Visvader and Lindeman, 2012), termed CSCs. According to the CSC model, cells are organized in a hierarchy where the CSC population is at the top and is capable of self-renewal as well as being able to differentiate into other cell populations. In the context of the brain tumor, this population has been identified as responsible for tumor initiation and as a driver of tumor growth (Galli et al., 2004; Ignatova et al., 2002; Singh et al., 2003; Singh 2014; Sotoriva et al., 2013).
et al., 2004). Additionally, CSCs can also drive therapeutic resistance to both chemotherapy (Chen et al., 2012) and radiotherapy (Bao et al., 2006). This subpopulation of tumor cells has been shown to grow as spheres in serum-free media supplemented with growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), self-renew, express normal neural precursor cell markers, and be able to recapitulate the original tumor when implanted intracranially in immunocompromised mice (Lathia et al., 2015). In order to study this cell population a critical step is its correct identification and isolation, which relies heavily in the use of differentially, expressed cell surface markers. Several markers have been used for the identification of CSCs such as prominin-1 (also known as CD133 (Singh et al., 2003)), integrin alpha-6 (Lathia et al., 2010), CD15 (Son et al., 2009), CD44 (Anido et al., 2010), EGFR (Jin et al., 2011), L1CAM (Bao et al., 2008), A2B5 (Ogden et al., 2008). While there are differences in the populations that can be isolated with each marker, CD133 remains the most commonly used. Nevertheless, it should be taken into account the fact that recent reports have shown that CD133- GBM cells also present with CSC features, such as sphere formation, self-renewal, and tumor initiation (Gambelli et al., 2012). These controversial results might be explained by low expression levels that further complicate their classification. Therefore when using CD133 as a CSC marker one must exert caution and validate results using additional markers when possible.
In general, the gold standard for CSCs is the capacity to initiate tumors in vivo, while being able to self-renew and have rapid rates of proliferation (Figure 1.3). Additional characteristics that have been observed but are not necessary to define them are the expression of stem cell markers such as Nestin, SOX2, OLIG2, their location in specific locations, and their ability to differentiate into multiple lineages (Figure 1.3). Identification and isolation of CSCs is only the first step towards studying this malignant population. While in vitro experiments can provide an understanding of the biology of the cells with the identification of upregulated or downregulated cellular pathways, CSCs do not work alone and to some extent, they are a result of their environment (which will be explored in the following section). The current lack of clinical efficacy can be boosted by therapies targeting CSCs specifically (Figure 1.4). In this scenario, current therapies, which will eradicate the bulk of the tumor, will work synergistically with CSC-targeted therapies to ensure the removal of all the malignant cells and prevent tumor recurrence.

1.4 Tumor microenvironment

As mentioned earlier, CSCs are not solely governed by internal programs but also influenced the tumor microenvironment that is essential in maintaining the balance between self-renewal and differentiation (Lathia et al., 2011). GBM CSCs are regulated by a variety of mechanisms that can be both intrinsic (genetics, epigenetics, and metabolism) and extrinsic (presence of niche factors, cellular microenvironment, and the host immune system) (Lathia et al., 2015) as
Figure 1.3. Functional characteristics require for the definition of CSCs. Although a variety of CSC markers exist, cells isolated should be able to initiate tumors in vivo, consistently proliferate and self-renew to be considered CSCs. Additional characteristics are composed of stem cell marker expression, location in specialized compartments, and multipotency. (Illustration assistance by Amanda Mendehlson, Medical Art Department, CCF.).
Figure 1.4. Targeting CSCs in combination with conventional therapies. Targeting only the bulk (top) or CSCs (middle) alone will have a temporary benefit for patients but the tumor will eventually grow back. For complete eradication, all cells in composing the tumor must be targeted. (Illustration assistance by Amanda Mendehlson, Medical Art Department, CCF).
seen in Figure 1.5. Pertaining to niche factor regulation, in resemblance to developmental programs, CSCs can be maintained in a non-differentiated state with factors that stimulate signaling pathways such as Notch, bone morphogenetic proteins (BMPs), mitogen-activated protein kinase (MAPK), and Wnt (Day et al., 2013; Li et al., 2009c; Rheinbay et al., 2013; Yan et al., 2014). The activation of these pathways is a result of both epigenetic and genetic properties of the cells as well as environmental cues present in their niche. The importance of such factors is not only in the upregulation of signaling cascades but also in the response and resistance to therapeutic challenges such as radiation or chemotherapy. Similarly, when talking about the cellular microenvironment one must envision a particular physical location where cells can interact with one another as well as with cells surrounding them. The existence of distinct niches is controversial however evidence has been reported supporting at least two independent niches: a perivascular (Gilbertson and Rich, 2007) and a hypoxic niche (Heddleston et al., 2009; Li et al., 2009b). In the first case, easy access to a blood vessel allows cells to secure oxygen and exchange of signals necessary to maintain their growth. Moreover, CSCs can secrete vascular endothelial growth factor (VEGF) that stimulates vasculature growth (Ebnet et al., 2004) while endothelial cells express notch ligands that stimulate pathways necessary for CSC maintenance. Conversely, hypoxia, which can arise as a result of necrosis and due to a lack of vasculature, not only promotes resistance to radiotherapy but can also facilitate the acquisition of stem like characteristics (Heddleston et al., 2009) due to upregulation of hypoxia inducible
Figure 1.5. Regulation of CSCs. Cell-autonomous (intrinsic) and external (extrinsic) forces regulates the CSC state. Key intrinsic regulators include genetic, epigenetic, and metabolic regulation, while extrinsic regulators include interaction with the microenvironment, including niche factors and the immune system. (Taken from Lathia, J.D. et al., Genes and Development, 2015).
factors (HIFs) that have been linked to activation of CSC self-renewal programs (Soeda et al., 2009). Thus, the cellular microenvironment is not just a physical location but it also encompasses all the potential cell to cell, and cell to environment interactions that can occur. This is particularly important because these interactions might not take place in a distinct area but can happen at any point. This is not difficult to imagine given that one of the key histological features of GBM is an increased cellularity where the probability of cells communicating with each other is very high.

1.5 Adhesion
One of the critical components of many tumor microenvironments is the extracellular matrix (ECM). The ECM is a collection of extracellular molecules secreted by cells in order to provide structural and biochemical support to surrounding cells. This structure provides a platform for diverse cellular processes such as cell-to-cell communication, adhesion, and differentiation; that will only take place if the right cells and signaling are cues are present at the same time. It is important to mention that while the existence of an ECM will modify a cell, the communication is bidirectional in the sense that cells can secrete factors or form interactions that can also modify the ECM, even under physiological conditions. With this in mind, one can imagine that tumor microenvironments possess a specialized ECM, one that can promote malignant processes such as invasion (Hu et al., 2008). In addition to binding several receptors, integrins act as receptors for many ECM ligands, such as laminins,
fibronectin, collagens, and vitronectin) and thus have a role in signal transduction. In this context, interaction of integrins with ECM components has two roles: first, the physical adhesion of cells to the ECM and second, the activation of intracellular programs dependent on the interaction. Integrins will therefore connect the ECM to the cytoskeleton (mainly actin filaments) of the cells expressing them. This interaction will allow cells to have a feel of their environment and react according to adverse or beneficial scenarios by regulating their cell cycle, differentiation, and survival pathways. Thus, adhesion is one of the main mechanisms by which cell to ECM communication can promote survival and self-renewal pathways.

In terms of cell to cell communication there are several adhesion molecules that can form these interactions like calcium-dependent integrins and cadherins and calcium-independent immunoglobulin superfamily cell adhesion molecules (CAMs), which include junctional adhesion molecules (JAM), receptor tyrosine phosphatase mu PTPmu, intercellular CAM (ICAM), neural CAM (NCAM), and L1CAM (Crossin and Krushel, 2000; Ensslen et al., 2003; Xu and Jin, 2010). Although the function of these has been characterized under physiological conditions, not much is known about their role modulating importance cell fate decisions in CSCs (Hale et al., 2012); see Figure 1.6. For instance, cadherins encompass a glycoprotein family of transmembrane molecules that can regulate cell-cell interaction, migration and signaling (Nollet et al., 2000). Cadherins, localized mainly in adherens junctions that bind cells within tissues together, also
Figure 1.6. CSC adhesion and communication. Schematic summarizing cell adhesion and communication mechanism between cell types in the tumor microenvironment and their role in CSC phenotypes. (Taken from Hale, J.S. et al., Cell Adhesion and Migration, 2012).
have intrinsic signaling capabilities and can activate several pathways important for self-renewal and cell survival such as Wnt (Kam and Quaranta, 2009), FGF (Williams et al., 2001), and AKT/PI3K (De Santis et al., 2009). Pertaining to tumor progression, dysregulation of cadherins has been shown to promote epithelial to mesenchymal transition (EMT); without a physical bond, tumor cells are free to migrate, proliferate, and colonize in new locations. However, cadherins have also been shown to regulate CSC quiescence in prostate and breast oncogenic contexts (Bae et al., 2011; Berx and Van Roy, 2001). Similarly, JAMs are mainly localized in endothelial tight junctions and are important for the extravasation of leukocytes and endothelia-platelet interaction. This group of molecules will be the focus of one of the studies presented in this dissertation and their role will be explored in subsequent sections. In summary, adhesion is critical for many cellular functions including survival, proliferation, and migration. Adhesion is also essential for CSC maintenance, as it promotes the abovementioned functions as well as localization to the niche and its instructive signaling. Therefore, the ability of a cell to acquire or lose adhesion mechanisms can result in it transitioning into or out of the stem cell state; consequently, adhesion can be considered a hallmark of stemness. While adhesion molecules such as integrins and Id proteins have been successfully targeted in GBM (Carbonell et al., 2013; Lathia et al., 2010; Niola et al., 2013), these same receptors are expressed in neural progenitor cells (Niola et al., 2012) and prove challenging for clinical translational approaches.
1.6 microRNAs

Gene expression can be regulated in several ways and at different times before the translation of a functional protein. At the transcriptional level, chromatin and epigenetic modifications can increase or decrease the accessibility of DNA to transcriptional machinery. Similarly, post-transcriptionally, RNA binding proteins (RBPs) or microRNAs (miRNAs) can bind to the messenger RNA (mRNA), once it has been synthesized, in order to alter its stability or target it for degradation. miRNAs are endogenous approximately 23 nucleotide non-coding RNAs that mediate post-transcriptional silencing (Bartel, 2009). miRNA biogenesis starts with transcription by RNA polymerase II which produces a pri-miRNA that is usually several kilobases long and can exceed 10 kilobases (Lee et al., 2006). This product requires additional processing to become a mature miRNA. First, still in the nucleus, the pri-miRNA will be cropped by a member of the ribonuclease III family (RNase III) named Drosha. After exiting the nucleus, another RNASE III, Dicer, catalyzes the final step yielding a miRNA duplex of around 23 nucleotides (Figure 1.7). One strand of this mature miRNA will be loaded onto the RNA induced silencing complex (RISC), which is mainly composed of Argonaute proteins (Joshua-Tor, 2006). When the complex is assembled, miRNAs can target transcripts for repression by mRNA deadenylation and posterior decay or translation blockade, the details of which are still being studied (Iwakawa and Tomari, 2015). It is important to mention that miRNAs are capable of silencing more than one mRNA due to imperfect complementarity used to bind to their targets. In fact, hundreds of mRNAs can be
Figure 1.7. Model for miRNA biogenesis. miRNA genes are transcribed by a RNA polymerase II to generate the primary transcripts (pri-miRNAs). The initiation step (cropping) is mediated by the Drosha–DGCR8 complex (also known as Microprocessor complex). Drosha, as well as DGCR8, is located mainly in the nucleus. The product of the nuclear processing is ~70-nucleotide pre-miRNA, which possesses a short stem plus ~2-nucleotide 3’ overhang. This structure may serve as a signature motif that is recognized by the nuclear export factor, Exportin 5 (Exp5). pre-miRNA constitutes a transport complex together with Exp5 and its cofactor Ran (the GTP-bound form). Upon export, the cytoplasmic RNase III Dicer participates in the second processing step (dicing) to produce miRNA duplexes. The duplex is separated, and usually one strand is selected as the mature miRNAs, whereas the other strand is degraded. In Drosophila, R2D2 forms a heterodimeric complex with Dicer and binds to one end of the siRNA duplex. It thereby selects one strand of the duplex. (Taken from Lee, Y. et al., Cold Spring Harbor symposia in quantitative biology, 2006).
affected by the same miRNA and this allows cells to regulate the expression of
genues that can regulate key cellular processes during development or tissue
repair. Similarly, it would not be surprising to find a dysregulation of miRNAs that
regulate these processes in the oncogenic context. For instance, a miRNA that
suppresses self-renewal and proliferation could be down regulated in malignant
cells leading to tumor growth and progression. Indeed, several studies have
demonstrated altered miRNA expression profiles in tumors where a CSC
population has been described such as GBM, prostate, and breast (Gonzalez-
Gomez et al., 2011; Liu, 2012; Majid et al., 2012). Yet, most of the downstream
targets of these miRNAs and the pathways they control remain to be elucidated.
In GBM, some miRNAs have been proposed to be pro-oncogenic as they are
overexpressed such as miR-21 (Conti et al., 2009), miR-26a (Huse et al., 2009),
miR-10b (Guessous et al., 2013), and miR-10a (Lang et al., 2012). Conversely,
other miRNAs might act as tumor suppressors and have been reported to be
downregulated in GBM such as miR-7 (Kefas et al., 2008), miR-34a (Li et al.,
2009a), and miR-125b (Wu et al., 2012). While downregulation or overexpression
of these miRNAs has been successful at reducing proliferation and self-renewal
of GCM CSCs, their expression is also shared with other normal brain cell types
such as astrocytes, neurons, and neural progenitor cells (NPCs). Therefore, the
correct characterization of the pathways they each control will be beneficial for
the understanding of these key molecular regulatory mechanisms and for the
development of CSC-specific therapies.
1.7 Linking immune signaling to self-renewal

Innate immunity has evolved as the front-line cellular defense mechanism to acutely sense and decisively respond to micro-environmental alterations. The toll-like receptor family activates signaling pathways in response to stimuli and is well-characterized in both resident and infiltrating immune cells during neural inflammation, injury, and degeneration. Innate immune signaling has also been observed in neural cells during development and disease, including in the stem and progenitor cells that build the brain and are responsible for its homeostasis. Recently, the activation of developmental programs in malignant brain tumors has emerged as a driver for growth via CSCs. In this section, I will discuss how innate immune signaling interfaces with stem cell maintenance in the normal and neoplastic brain.

The Toll receptor was first described in Drosophila, where it is essential for the establishment of dorso-ventral patterning during development (Hashimoto et al., 1988). Toll is similarly involved in the control of antifungal responses in the adult fly (Lemaitre et al., 1996). The first Toll receptor homolog was later identified in humans (Medzhitov et al., 1997), and eventually a family of Toll-like receptor (TLR) proteins was described, the major function of which is to mediate recognition of both pathogen and damage-associated molecules. The TLR family now consists of ten members in humans (TLR1-TLR10). The cytoplasmic region of all TLRs is very similar to that of the interleukin-1 receptor family, and is known as the Toll/IL-1 receptor (TIR) domain (Takeda and Akira, 2015). Despite this
common feature, TLRs are capable of recognizing different molecular patterns to elicit a response. While the role of TLRs has been well characterized in the innate immune system, there are several additional functions that have not been completely understood or explored, such as their function during development, which was the original observed role of the *Drosophila* Toll receptor. Below, I will examine recent evidence highlighting the importance of TLRs in development in both physiological and malignant backgrounds. The identification of downstream molecules mediating the differential function of TLR signaling in normal and neoplastic cells will be critical for the design of future therapeutic strategies.

Although the role of TLRs has been well studied in immune processes, TLR expression is not exclusively limited to immune cells (i.e., macrophages, dendritic cells, neutrophils, T cells, and B cells) but has also been reported in multiple other cell types under physiological conditions including nervous (Jack et al., 2005; Okun et al., 2009; van Noort and Bsibsi, 2009), muscular (Beswick et al., 2014; Xu et al., 2015), reproductive (Aflatoonian and Fazeli, 2008; Hu et al., 2015), colonic (Niedzielska et al., 2009), adipose (Fusaru et al., 2012; Nativel et al., 2013), renal (Gluba et al., 2010; Liu et al., 2012), hepatic (Saito et al., 2011), and alveolar (Yang et al., 2014) tissue. A complete tissue-specific mRNA expression profile of human TLRs can be found in (Nishimura and Naito, 2005). This diverse expression is not surprising based on the description of TLRs as receptors for not only pathogen-associated molecular patterns (PAMPs) but also endogenous damage-associated molecular pattern (DAMP) ligands. DAMPs are
key danger signals that are released in response to tissue damage that initiate a 
repair process (Piccinini and Midwood, 2010) However, DAMPs can also play a 
role in the progression of pathogenesis by stimulating inflammation and releasing 
cytokines. This is true in both autoimmune diseases such as rheumatoid arthritis 
(Taniguchi et al., 2003) or multiple sclerosis (Andersson et al., 2008) and cancer, 
where necrosis is a hallmark of malignant progression (Orend and Chiquet-
Ehrismann, 2006; Rybarczyk and Simpson-Haidaris, 2000). Perhaps the most 
remarkable feature of this function of TLRs is the ability for the same receptor to 
recognize, despite their diversity, several endogenous ligands (proteins, fatty 
acids, or degradation products of the ECM, listed here (Piccinini and Midwood, 
2010)). This promiscuity can be explained by the fact that TLRs can exist in the 
membrane in the presence of several different co-adaptor molecules that are 
able to modulate the interaction with various ligands, thereby providing 
specificity. Therefore, the capacity of cells to respond to several stimuli in a 
physiological setting can be transposed to the involvement of TLRs in several 
malignancies. For example, there has been an emphasis on studying TLR4 
signaling based on its association with several diseases including 
neurodegeneration, traumatic brain injury, Alzheimer’s disease, multiple 
sclerosis, Parkinson’s disease, and amyotrophic lateral sclerosis (Trotta et al., 
2014).

In the setting of the brain, an organ that has unique and specialized immune 
response mechanisms, TLRs have been described in resident, non-immune
neural cell types including microglia, astrocytes, oligodendrocytes, neurons, and NPCs (Jack et al., 2005; Okun et al., 2009; van Noort and Bsibsi, 2009). Both microglia and astrocytes express virtually all known TLRs, while oligodendrocytes, neurons, and NPCs express TLR2, TLR3, and TLR4 (Table 1.1). Of note is the expression of TLR8 in both neurons and NPCs. The differential expression of TLRs in distinct cellular populations is interesting and deserves detailed exploration considering the fact that microglia are the only resident cell type thought to serve immune-related functions in the brain (Shemer et al., 2015). Additionally, while the presence of a particular receptor subtype is capable of mediating the response to a specific ligand, selective recruitment of adaptor proteins and downstream effector activation contribute substantially to the receptor-mediated response to certain ligands. As reviewed by (Okun et al., 2011), TLR4 signaling in neural cells is different from the canonical dendritic cell signaling (Hemmi and Akira, 2005) where stimulation by lipopolysaccharide (LPS), a TLR4 ligand, leads to activation and translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (in a myeloid differentiation primary response gene 88 (MyD88)-dependent manner) that ultimately results in increased expression of tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and interleukin 12 (IL-12); TLR4 signaling is similarly capable of inducing interferon regulatory factor (IRF)-3 activation (which is MyD88-independent). Microglia and astrocytes exhibit comparable TLR4 signaling, yet astrocytes are unable to activate IRF-3 (Gorina et al., 2011). Moreover, TLR4 activation in neurons does not appear to induce the canonical downstream
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Table 1.1. TLR receptor expression in neural cells.
signaling and different adaptor molecules are present in the membrane, i.e., MD1 instead of MD2 as in all other cell types (Jung et al., 2005). Similar differential responses were observed when human microglia and astrocytes were stimulated with synthetic lipopeptide (PAM), synthetic dsRNA polyinosinic:polycytidylic acid (PolyI:C), and *E. coli* LPS (Jack et al., 2005). In the case of microglia, both PolyI:C and LPS are able to induce significant levels of TNF-α while all three stimuli are capable of eliciting IL-6 release. In contrast, astrocytes were only responsive to stimulation of TLR3 with PolyI:C, which led to the robust secretion of IL-6; however, TNF-α was undetectable. These observations further underscore the importance of downstream effector selection in the presence of various stimuli under physiological conditions.

In the context of NPCs and neural development, there is evidence that TLRs are involved in proliferation (Lathia et al., 2008; Okun et al., 2010; Rolls et al., 2007; Trotta et al., 2014), differentiation (Bsibsi et al., 2006; Rolls et al., 2007; Sloane et al., 2010), and survival/migration (Bsibsi et al., 2006; Ma et al., 2007; Ma et al., 2006) at distinct developmental stages. In the adult mammalian brain, deficiency of TLR4, MyD88, or TRIF increases the proliferation of dentate gyrus-derived NPCs, yet this effect is not recapitulated by either TLR2 or TLR3 deficiency (Okun et al., 2011). Additionally, stimulation with LPS (for TLR4) or PolyI:C (for TLR3) inhibits adult NPC proliferation (Covacu et al., 2009; Okun et al., 2010; Rolls et al., 2007). More recently, TLR9 stimulation with CpG oligodeoxynucleotide (ODN) in NPCs resulted in the release of neuroprotective
molecules such as the chemokine receptor CX3CR1 and triggering receptor expressed on myeloid cell-s (TREM2). This in turn switches microglia from a pro-inflammatory to an anti-inflammatory, protective phenotype (Wu et al., 2014). Although some of the endogenous ligands that bind these receptors to activate downstream effectors have been described, there is still room for exploration in this regard. Nevertheless, the expression of TLRs in distinct adult populations clearly emphasizes their role in neural cells to achieve normal homeostasis and proper function.

1.8 TLRs in the oncogenic context

The link between development and disease has been well established, and tumor progression is often dependent on the utilization of key developmental pathways. The diversity and function of TLRs have been associated with tumor development and progression in several malignancies including breast (Bhatelia et al., 2014; Yang et al., 2014; Yusuf, 2014), colon (Grimm et al., 2010; Li et al., 2014; Lu et al., 2015), pancreas (Santoni et al., 2015; Vaz and Andersson, 2014), prostate (Galli et al., 2010; Zhao et al., 2014), liver (Huang et al., 2012), lung (Chatterjee et al., 2014; Ke et al., 2015), leukemia (De Luca et al., 2009; Ignatz-Hoover et al., 2015; Morrison et al., 2011), and ovarian (Husseinzadeh and Davenport, 2014; Szajnik et al., 2009) cancers, among others. In general, independent of the specific TLR protein, activation of these receptors is linked to the recruitment of immune cells and the release of pro-inflammatory cytokines. This, in turn has been associated with a favorable environment for tumorigenic
cells to thrive and disproportionately proliferate. Although expression of most TLRs has been shown for the above-mentioned tumors using immunohistochemistry and RNA screening techniques, the expression of TLRs is not necessarily associated with an oncogenic setting. This family of receptors has also been studied in different situations. For example, in breast cancer, expression of all TLRs has been examined (Bhatelia et al., 2014; Yang et al., 2014), but only TLR4 was targeted. Decreased TLR4 inhibited proliferation and survival of breast cancer cells in vitro (Yang et al., 2014). Furthermore, TLR7 and TLR8 expression was examined together with that of CD133, a canonical marker of self-renewing cancer stem cells, and these two TLRs were found to also serve as CSC markers in colorectal cancer (Grimm et al., 2010). TLR1, TLR2, TLR4, and TLR8 were also found to be higher together with downstream targets such as IL-6 in colorectal cancer patient tissue compared with normal mucosa (Lu et al., 2015). It is of paramount importance to carefully study where these TLRs are expressed and the downstream cascades that are activated to increase our understanding of TLR function and develop efficient therapeutics. In the following sections, I will focus on TLRs in brain tumors and, specifically, in individual cell populations where TLRs are involved in a myriad of malignant processes such as invasion, migration, proliferation, and immunosuppression. Lessons on the function of TLRs in normal brain development and function may be leveraged to identify key regulatory mechanisms for tumor growth and progression that could ultimately be targeted.
As mentioned earlier, GBM is the most common malignant primary brain tumor in adults. In addition to malignant cells, the tumor microenvironment is also composed of tumor-infiltrative cells and endothelial cells that together with the physical location of the tumor contribute to regulating the function of CSCs. GBM has been a prototypic tumor for the development and testing of novel targeted therapies (including specific pathway inhibitors and agents targeting key biological processes, such as angiogenesis (Alexander et al., 2015; Prados et al., 2015)). Despite promising pre-clinical and early-stage clinical trial success, these approaches have translated to limited therapeutic efficacy. Recently, there have been promising advances in immunotherapeutic approaches for GBM treatment, many of which have been inspired by the success in other tumor types such as melanoma (Larkin et al., 2015). These approaches encompass cellular (adoptive transfer or chimeric antigen receptor T cells and bispecific T-cell engagers), vaccination (tumor-specific antigens such as epidermal growth factor receptor vIII and tumor-associated antigens such as ephrin type-A receptor 2 (EphA2) or interleukin-13 receptor subunit alpha-2 (IL13Ra2)), and immunomodulatory (programmed death 1 (PD1), PD1 ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated protein 4 (CTLA4) blockade) techniques (Reardon et al., 2014).

Stimulation of the innate immune system with the goal to boost the eradication of tumor cells is part of the novel efforts of glioma immunotherapy. Based on their role in the immune response, TLRs are likely candidates for adjuvant therapies.
Indeed, several clinical trials are at various stages that are using TLR ligands for this purpose (most are still in the patient recruitment stage; summarized in (Deng et al., 2014). However, as in the normal brain where non-immune cells utilize TLRs for a variety of functions, the expression of TLRs on tumor cells has not been exhaustively explored, and TLR stimulation can lead to pro-tumor effects if caution is not applied. In this context, TLR9 has been found to be elevated in GBM CSCs (Herrmann et al., 2014). In patients, high TLR9 expression is associated with poorer survival compared with low expression in several cancers including GBM (Leng et al., 2012; Meng et al., 2008). When CSCs were treated with the TLR9 ligand CpG-ODN, an induction in Janus kinase 2 (JAK2) activation was observed in a Frizzled 4-dependent manner (Herrmann et al., 2014). This in turn could lead to the induction of signal transducer and activator of transcription 3 (STAT3), a known critical transcription factor for CSC maintenance (Guryanova et al., 2011; Kim et al., 2013; Sherry et al., 2009). TLR4 expression has also been reported in glioma cell lines (Gupta et al., 2013; Sarrazy et al., 2011; Tewari et al., 2012). When TLR4 signaling was disrupted in glioma cells treated with TNF-α, there was a decrease in the induction of the transcription factors IRF3 and signal transducer and activator of transcription 1 (STAT1) together with interferon (IFN) beta and inflammatory cytokines (Tewari et al., 2012). Additionally, treatment of cell with interleukin 1 (IL1)-beta induced expression of the non-classical human leukocyte antigen (HLA) class I antigen HLA-G and TLR4 in a hypoxia inducible factor (HIF)-1 alpha-dependent manner. This is relevant to CSC biology as HIFs have been reported to be associated with CSC
maintenance and tumor progression (Heddleston et al., 2010; Li et al., 2009b; Soeda et al., 2009). Interestingly, beta-defensin 3, the expression of which has been found to be elevated in GBM specimens, prevented the signaling of this pathway and the release of pro-inflammatory mediators (Gupta et al., 2013). Moreover, treatment with either Fas ligands or LPS led to increased cell proliferation; however, when both ligands were combined, there was an anti-proliferative effect with a concomitant decrease in cell migration and matrix metalloprotease (MMP)-9 expression (Sarrazy et al., 2011). These data demonstrate the complex role of TLR family members in GBM.

In the tumor microenvironment, which is enriched in resident and infiltrating immune cells, glioma cells are not the only cells that express TLRs. In this sense, the regulation of tumor progression and CSC maintenance could be indirectly driven by TLR signaling in these immune cell populations. Certainly, resident microglia and macrophages present in the tumor niche are capable of interacting with CSCs to regulate their tumorigenic potential. For example, stimulation of TLR2 on the surface of microglia induced membrane type 1 (MT1)-MMP expression (Vinnakota et al., 2013). Likewise, intracranial injection of GL261 cells in a TLR2 KO background yielded smaller tumors. In a similar study, (Hu et al., 2014) it was demonstrated that glioma supernatant upregulates the expression of TLR2 in microglia with the concomitant expression of MMP9; these effects can be suppressed by the antibiotic minocycline and prolong survival in glioma-bearing mice. This further underscores the importance of metalloproteases for
the degradation of the ECM during glioma invasion as previously reported (Markovic et al., 2009). It would be interesting to examine if the roles of TLRs during development and adult neurogenesis, in microglia, astrocytes, and NPCs, are associated with GBM tumor initiation and progression. Although limited, the study of cells that express TLRs in the glioblastoma tumor microenvironment has shed light on new interactions that could be explored for future therapeutics.

The evidence presented thus far delineates important roles played by TLRs in microglia, astrocytes, neurons, NPCs, and tumor cells in both physiological and malignant settings discussed in this section (Figure 1.8). Although the role of TLRs in normal homeostasis in the adult brain is well defined, their involvement has not been carefully characterized in the context of brain tumors, which share hallmarks of development including a self-renewing stem cell population.

1.9 Experimental approach to CSCs
As mentioned earlier, the first step for the study of CSCs is the identification and isolation of this cell population. For this purpose, our laboratory utilizes CD133 as a CSC marker that has been validated with several cells derived from tumor specimens from GBM patients. All protocols and procedures used have been approved by Cleveland Clinic Internal Review Board and Institutional Animal Care and Use Committee. The specimens obtained during surgery are dissociated and passaged through immunocompromised mice (NSG obtained from The Jackson Laboratories) by injecting them into the flank. This is done in
Figure 1.8. TLRs in the normal brain and glioma tumor microenvironment. (Left) In the normal brain, stimulation of neural progenitor cells (NPCs) with both lipopolysaccharide (LPS; TLR4 ligand) and polyinosinic:polycytidylic acid (PolyI:C; TLR3 ligand) leads to a decrease in proliferation while stimulation with CpG oligodeoxynucleotides (CpG; TLR9 ligand) induces secretion of neuroprotective factors CX3CR1 and TREM2. For microglia, stimulation with LPS, PolyI:C, and synthetic triacylated lipopeptide (PAM; TLR2 ligand) induces secretion of interleukin (IL)-6 but only LPS and PolyI:C promote secretion of tumor necrosis factor (TNF)-α. Similarly, astrocytes only respond to PolyI:C and secrete IL-6 but not TNF-α. (Right) In contrast, LPS treatment of cancer cells promotes proliferation and induces secretion of matrix metalloprotease (MMP)-9. TLR9 has also been found to be elevated in cancer cells. Stimulation of TLR2 with PAM leads to secretion in MT1-MMP, which is critical for extracellular matrix degradation in glioblastoma. The presence of damage associated molecular patterns (DAMPs) and which cells can these stimulate requires further investigation. This panel also shows the presence of blood vessels (red) in the tumor microenvironment. (Illustration assistance by Amanda Mendehlson, Medical Art Department, CCF).
order to obtain more cells and to maintain the heterogeneity from a limited amount of tissue that is not easily accessible. Once these xenografts exceed 5% of the animal's body weight, the mice are sacrificed and the tumors are once again mechanically dissociated using a papain dissociation kit (Worthington Biochemical Corporation). Cells are culture overnight in neurobasal medium (Life technologies) supplemented with B27 (Life technologies), sodium pyruvate, L-glutamine, EGF (20 ng/mL) and FGF-2 (20 ng/mL) (R&D Systems). The day after CSCs are isolated using CD133/2 magnetic beads (Miltenyi Biotech) and cultured in the supplemented neurobasal medium. The non-stem cells (non-CSCs) are also collected during the magnetic separation. These cells are cultured in parallel in DMEM supplemented with 10% fetal bovine serum (Sigma). Both tumor cell populations are kept on culture as used as direct control to each other; only low (<5) passage cells were used for experiments to prevent cellular drift. Our belief is that this approach allows us to compare cell populations that were obtained from the same GBM specimen in order to discover cellular pathways that are differentially express in CSCs as compared with non-CSCs. Importantly, only CSCs are capable of initiation tumors in vivo that recapitulate the original malignancy, when injected intracranially (Figure 1.9). With this model, cells can be obtained from a tumor and used to study signaling pathways in vitro or can be treated with specific agents and then used in vivo to test if the pathways and molecules identified in vitro have functional consequences.

1.10 Outstanding questions
Figure 1.9. Experimental approach for CSC study. Cells obtained in the clinical side are passaged through mice in order to expand the malignant cells and maintain the heterogeneity. CSCs are isolated using the CD133 surface marker. Both CSCs and non-CSCs are cultured under specific media condition in order to ensure their survival. Distinct cell populations from the same tumor are used as controls for each other. CSCs, when intracranially transplanted, can form tumor that recapitulate the original malignancy.
CSCs can be regulated by components of the tumor microenvironment. One of these is the expression of cellular adhesion molecules (that facilitate cell-to-cell as well as cell-to-ECM interactions). Previous work has linked the presence of integrins and adhesion molecules to CSC maintenance however the mechanisms that govern the expression of these critical proteins have not been explored. Similarly, the link between TLR signaling and self-renewal has been covered extensively however key question pertaining to its role in CSC remain unanswered. Mainly, which TLRs are being expressed and whether their activation is beneficial or detrimental to the cells expressing them. Although I am studying two distinct signaling pathways, the overall goal is to understand them in the context of CSCs and GBM. Therefore, the overall hypothesis of this dissertation is that targeting (either by activation or inhibition) stem cell pathways in CSCs reduces self-renewal and tumor formation and has the potential to be translated into clinical approaches.

1.11 Research Objectives

To investigate the regulatory mechanism by which adhesion molecules are highly expressed in CSCs I used a previously reported CSC adhesion molecule (Lathia et al., 2014), JAM-A, as a model to investigate micro-RNA regulatory networks. In chapter 2, I describe the discovery of a specific miRNA that is downregulated in CSCs and has dramatic effects on proliferation, self-renewal and tumor formation when restored into the system.
To address the expression of TLRs and their role in CSC maintenance I conducted several studies aiming to examine how tumor cell populations respond to the stimulus with different TLR ligands. In chapter 3, I describe the downregulation of a member of the TLR family and the detrimental effects it can have when activated in GBM cancer cells. I will also describe studies that reveal a non-canonical role of TLRs linking innate immune signaling to regulation of histone modification proteins and transcription activation.
Chapter 2: JAM-A / miR-145 axis regulates GBM self-renewal
2.1 Introduction

Cellular heterogeneity is recognized as a hallmark of advanced tumors, and this phenotype has recently been appreciated as a contributing factor in the complexity of cancer. Functional studies have sought to determine how distinct cell populations contribute to tumor growth and therapeutic resistance. These efforts have led towards the identification of a population of tumor cells with enhanced self-renewal and tumor-initiation capacities that possess stem cell-like features termed cancer stem cells (Reya et al., 2001). CSCs are a dynamic population that is maintained in discrete anatomical niches that promote their self-renewal, tumor maintenance and increased resistance to conventional therapies (Visvader and Lindeman, 2012). GBM, the most prevalent malignant primary brain tumor, contains CSCs (Galli et al., 2004; Hemmati et al., 2003; Ignatova et al., 2002; Singh et al., 2003; Singh et al., 2004) and has been a prototypic tumor for the study of CSC biology. Despite aggressive treatment consisting of maximal safe surgical resection, radiation, and chemotherapy, 5-year overall survival for GBM patients is less than 10% (Stupp et al., 2009; Stupp et al., 2005). Identification and subsequent targeting of mechanisms responsible for the maintenance of CSCs in combination with current GBM treatments may have a synergistic therapeutic effect and therefore improve patient prognosis.

To fulfill the eventual goal of developing CSC targeted therapies, the identification of CSC-specific regulatory mechanisms is required. Based on the importance of niche interactions to CSC maintenance, interrogating signaling
mechanisms present within the niche remains a priority. In a manner similar to the dependence of neural progenitor cells (NPCs) on extracellular interactions with their microenvironment (Loulier et al., 2009; Niola et al., 2012), the interaction of CSCs with their niche via adhesion proteins is critical for their maintenance in GBM (Lathia et al., 2010; Lathia et al., 2012; Niola et al., 2013). While adhesion molecules involved in CSC-niche interactions have been identified that drive CSC maintenance and therapeutic resistance in GBM (Lathia et al., 2010; Lathia et al., 2012; Lathia et al., 2014; Niola et al., 2013), the molecular circuitry responsible for the regulation of adhesion molecules and how they are integrated into larger signaling networks has yet to be determined. Multiple phenotypes in GBM, including tumor suppression (Kefas et al., 2008), therapeutic resistance (Corsten et al., 2007), and self-renewal (Kefas et al., 2008; Lopez-Bertoni et al., 2014; Peruzzi et al., 2013; Siebzehnrubl et al., 2013), have been linked to miRNAs. However, the interaction between miRNAs and niche adhesion molecules remains largely unexplored. Using junctional adhesion molecule A (JAM-A), a CSC-specific adhesion protein, as a paradigm, I sought to identify the miRNA regulatory circuitry linking niche adhesion molecules to a larger signaling network. Through a screen for miRNAs that bind to JAM-A and are downregulated in GBM, I identified miR-145 as a negative regulator of JAM-A-mediated CSC maintenance.
2.2 Materials and Methods

2.2.1 In vitro functional analysis

In order to functionally study CSCs in vitro two experimental approaches have been utilized. The first test measures proliferation of cells under different conditions and after genetic modifications. In this manner, cell growth can be assessed which represents one of the key defining concepts of CSCs. The second test is an in vitro surrogate of self-renewal. By plating low number of cells and measuring their capacity to grow into spheres we can assess their true stem cell potential. Cell proliferation experiments were conducted by plating cells of interest at a density of 1000 cells/well in a 96-well plate in triplicate. Cell number was measured every other day and normalized to the initial reading at Day 0 using the CellTiter-Glo assay kit (Promega, Madison, WI, USA). For tumorsphere formation experiments, cells were sorted using a flow cytometer (FACS Aria II) into 96-well plates at a density of 1, 5, 10 and 20 live cells per well (24 wells for each density). Cells were maintained for 10 days before sphere formation was evaluated. Spheres larger than 10 cells in diameter were considered for analysis. Reported numbers represent either number of cells per well or stem cell frequency calculated using the Walter and Eliza Hall Institute Bioinformatics Division ELDA analyzer (http://bioinf.wehi.edu.au/software/elda/) (Hu and Smyth, 2009).

2.2.2 Immunoblotting analysis
Cell populations were lysed using RIPA lysis buffer (containing PMSF, protease inhibitor cocktail, and sodium orthovanadate; Santa Cruz Biotechnology, Dallas, TX, USA), and protein concentrations were calculated using a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). After denaturation with Laemmli buffer (BioRad Laboratories, Hercules, CA, USA), 10 µg of total protein was loaded on 12% polyacrylamide SDS-PAGE gels, transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and probed using the following antibodies: JAM-A (B&D Biosciences, San Jose, CA, USA, 1:1000), SOX2 (R&D Systems, 1:1000), AKT (Cell Signaling, Danvers, MA, USA, 1:2000), and p-AKT (Cell Signaling, 1:2000); β-Actin (Santa Cruz Biotechnology, 1:2000) was used as a loading control. Species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies were used for detection (Invitrogen, 1:5000). Membranes were developed using ECL-2 reagent (Pierce Biotechnology).

2.2.3 miRWalk database and sequence alignment
Using the predicted gene-miRNA interaction search, a list of candidate miRNAs was generated. Results were validated by comparison with other databases, and only those miRNAs present in an additional three databases were included in Figure 2.3. The sequence alignment between miR145 and JAM-A was generated using miRanda ((John et al., 2004); http://www.microrna.org).

2.2.4 Lentiviral shRNA and overexpression generation
Lentiviral constructs were prepared according to modified protocols from
Tronolab ([http://tronolab.epfl.ch](http://tronolab.epfl.ch)). In short, using calcium phosphate precipitation, 293FT cells were co-transfected with the packaging vectors psPAX2 and pMD2.G (Addgene, Cambridge, MA, USA) and lentiviral vectors directing the expression of i) MISSION shRNA (Sigma) specific to SOX2: (TRCN0000003252 (KD1) and TRCN0000003253 (KD2)) or a non-targeting control (NT) shRNA (SHC002) and ii) overexpression of JAM-A: accession number BC001533 (LV152204; Applied Biological Materials, Richmond, Canada) or control vector (LV590) to produce virus. Media on the 293FT cell cultures were changed 18 hours after transfection, and viral supernatants were collected 12, 24, and 36 hours later and concentrated using polyethylene glycol precipitation for immediate use or stored at -80°C for future use.

### 2.2.5 *In vivo* intracranial injections

For *in vivo* tumor formation, JAM-A or control vector-containing live CSCs were transplanted into the frontal lobe of NOD scid gamma (NSG) mice at 100 or 1000 cells per mouse (n=8). Mice were monitored daily and sacrificed upon the development of neurological signs.

### 2.2.6 miR-145 introduction

Approximately 2x10^6 cells were transfected with 20 pmol of miR-145 or NT control mimics (Dharmacon, Lafayette, CO, USA) utilizing an Amaxa Nucleofector II and the Mouse Neural Stem Cell Nucleofector Kit (Lonza, Basel, Switzerland) as previously described (Marchenko and Flanagan, 2007). The
transfected cells were then collected after 3 days and used for downstream analyses including immunoblotting and qPCR.

2.2.7 Luciferase expression

Cells containing NT control or miR-145 mimics were used for transfection with luciferase constructs. A total of 20,000 cells per condition were plated in triplicate in 96-well plates pretreated with Geltrex (Life Technologies), which was used as an adherence substrate. The next day, cells were transfected with luciferase only, luciferase + 3'UTR, or luciferase + 3'UTR without seed sequence (ACAATGGACCTTTTGAACTGGAA) constructs using Lipofectamine 2000 (Life Technologies) with 0.6 µg of DNA per reaction in Opti-Mem (Lerner Research Institute Media Core) medium. After 6 hours at 37°C, cells were washed, and luciferase levels were measured 48 hours later using Dual-Glo Luciferase Assay (Promega) per the manufacturer’s instructions.

2.2.8 qRT-PCR

RNA from cells of interest was extracted using TRIzol (Life Technologies), and cDNA was synthesized using the Superscript III kit (Invitrogen). qPCR reactions were performed using an ABI 7900HT system using SYBR-Green Mastermix (SA Biosciences, Valencia, CA, USA). For qPCR analysis, the threshold cycle (CT) values for each gene were normalized to expression levels of β-Actin. Dissociation curves were evaluated for primer fidelity, and only threshold cycles below 35 cycles were reported. A complete list of the primers used for these
experiments can be found in the Appendix I section.

For microRNA analysis, I used the Taqman MicroRNA Cells-to-Ct Kit (Applied Biosystems) per the manufacturer's instructions. miRNA-145 and control U6 snRNA 5x and 20x primers (PN4427975; Applied Biosystems) were used with this kit.

### 2.2.9 Patient database bioinformatics

Gene and miRNA expression data were obtained from The Cancer Genome Atlas (TCGA; [https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp](https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp)) for patients with GBM (Cancer Genome Atlas Research, 2008). Patients were divided into high and low groups based on mean +/- one standard deviation, respectively. Kaplan Meier survival curves were generated comparing these two groups via log-rank test. microRNA data were combined with expression data for JAM-A, NANOG or SOX2 by subtracting the expression data from the miR-145 data for each patient, and the analysis described above was repeated. Patients were also divided based on their molecular subtype and compared with each other using one-way ANOVA. Combined microRNA/gene expression analyses were also performed for each of the molecular subtypes.

### 2.2.10 Statistical analysis

Reported values are mean values +/- standard error of the mean from studies performed at least in triplicate. Unless otherwise stated, one-way ANOVA was
used to calculate statistical significance, with p-values detailed in the text and figure legends.

2.3 Results

2.3.1 JAM-A gain of function increases proliferation, self-renewal, and tumor initiation in vivo

Our laboratory previously demonstrated that JAM-A was essential for CSC maintenance (Lathia et al., 2014). To assess the sufficiency of JAM-A to drive CSC malignancy and aggressiveness, I evaluated the effect of JAM-A overexpression. JAM-A was overexpressed in CSCs using a lentiviral vector (JAM-A vector) to achieve stable transfection and led to an increase in proliferation compared with CSCs treated with control vector (Figure 2.1, panel a). Similarly, self-renewal was elevated in CSCs treated with the JAM-A vector as assessed by in vitro limiting dilution analysis (Figure 2.2); the stem cell frequencies generated showed an increase from 1:7, 1:11, and 1:17 to 1:3, 1:7, and 1:9 in JAM-A overexpressing CSCs compared with control vector, respectively, in the specimens analyzed. Likewise, this increase in proliferation was also observed in non-CSCs when JAM-A was overexpressed using the same lentiviral system (Figure 2.4, panel b). I evaluated several key signaling nodes in CSCs and found that JAM-A overexpression was associated with an increase in p-AKT levels and SOX2 expression (Figure 2.1, panel b and Figure 2.4, panel a). Next, I evaluated whether differences in tumor initiation and growth
Figure 2.1. JAM-A gain-of-function increases proliferation. JAM-A overexpression in CSCs increased proliferation (a). Immunoblots demonstrate that CSCs overexpressing JAM-A also have higher levels of p-AKT and SOX2 (b) than CSCs expressing a control vector. ** p<0.01, *** p<0.001 as assessed by one-way ANOVA.
Figure 2.2. JAM-A gain-of-function increases self-renewal. JAM-A overexpression in CSCs increased stem cell frequencies.
Figure 2.3. JAM-A gain-of-function increases tumor initiation in vivo. Median survival was significantly decreased from 42 to 34 days in mice intracranially injected with 1000 CSCs overexpressing JAM-A. The log-rank p-value for significance between groups is shown next to the survival curve.
Figure 2.4. JAM-A overexpression signals through AKT and increases proliferation in non-CSCs. Protein expression analysis in an additional specimen (T387) confirms JAM-A overexpression induces higher levels of p-AKT and SOX2 (a). Proliferation of non-CSCs is elevated in JAM-A-overexpressing cells compared with vector-expressing cells (b). * p<0.05, ** p<0.01 as assessed by one-way ANOVA.
could be seen *in vivo* by intracranially transplanting CSCs containing control or JAM-A vectors. The median survival of mice injected with 1000 cells per mouse was reduced from 42 days in the blank vector to 34 days in the JAM-A vector group (Figure 2.3). An equally significant decrease in median survival was seen when 100 cells per mouse were intracranially transplanted (62 days in the blank vector vs. 47 in the JAM-A vector group (a reduction of 24%; data not shown). These data verify the importance of JAM-A in CSC self-renewal and demonstrate that JAM-A overexpression drives CSC marker expression, proliferation, and self-renewal *in vitro* and tumor initiation *in vivo*.

### 2.3.2 JAM-A is a target of miR-145, which is downregulated in CSCs

CSCs do not operate alone but rather respond to and interact with components of their tumor microenvironment. As adhesion molecules are an essential part of this niche, I investigated the upstream regulatory mechanism behind JAM-A expression. To couple adhesion to a larger signaling network, I identified miRNAs that are predicted to bind to JAM-A using the miRWalk database (Dweep et al., 2011). I compared this list to miRNAs reported to be downregulated in GBM (Moller et al., 2013; Zhang et al., 2012) and found miR-145 as a potential target (Figure 2.5, panel a). I further validated these results in two independent GBM cohorts and found that miR-145 expression was decreased in GBM compared with neural progenitor cells (Figure 2.7, panel a) and non-neoplastic brain tissue (Figure 2.7, panel b). miR-145 has been previously associated with tumor suppressor functions in GBM via suppression of neural precursor cell expressed
Figure 2.5. JAM-A is a target of miR-145. Schematic of miRNAs predicted to bind to JAM-A generated with miRwalk and miRNAs reported to be downregulated in GBM (a), illustrating miR-145 (red) as a potential upstream regulator of JAM-A. Confirmation of binding by sequence alignment (b)
Figure 2.6. miR-145 is downregulated in CSCs. Luciferase expression (a and c) confirm JAM-A as a direct target of miR-145. miR-145 and JAM-A mRNA are reciprocally expressed in non-CSCs and CSCs (b and d). SOX2 and OLIG2 were used as controls and are higher in the CSC population. * p<0.05, ** p<0.01, *** p<0.001 as assessed by one-way ANOVA, NS represents not statistically significant.
Figure 2.7. miR-145 levels are lower in GBM. Human GBM specimens were analyzed for the expression of the miR candidates shown in Figure 2a. miR-145 is expressed at lower levels in GBM spheres and GBM tissue compared with neural progenitor cells (a) and non-neoplastic brain tissue (b).
developmentally downregulated 9 (NEDD9), a scaffolding protein involved in invasion (Speranza et al., 2012). Sequence alignment suggested that miR-145 binds to the 3'UTR region of the JAM-A mRNA to block its translation or promote its degradation (Figure 2.5, panel b). I therefore used the 3'UTR region of JAM-A in luciferase assays to confirm that JAM-A is a direct target of miR-145. In CSCs where I introduced a control non-targeting microRNA (NT mimics), I observed that both the luciferase control and the luciferase construct with JAM-A 3'UTR were expressed at similar levels (Figure 2.8). However, when miR-145 was introduced (miR-145 mimics), I observed a decrease in expression in only the luciferase with JAM-A 3'UTR construct (Figure 2.8). To further confirm that the difference observed was due to miR-145 binding to the putative binding site in the JAM-A 3'UTR, I also introduced constructs without the seed binding region (mut) in CSCs. Luciferase levels were comparable in the mut and complete 3'UTR constructs when NT mimics were introduced in both specimens analyzed (Figure 2.6, panels a and c). However, upon miR-145 introduction, only the complete 3'UTR evidenced a decrease in luciferase, while the construct lacking the binding region to miR-145 showed no change (Figure 2.6, panels a and c). To assess whether the endogenous expression levels of miR-145 were lower in CSCs compared with their non-CSC counterparts, the expression levels in freshly dissociated xenograft tumors were evaluated after enrichment for CSCs using CD133 as a surface marker. In all specimens analyzed, miR-145 levels were significantly lower in the CSC population (Figure 2.6, panels b and d and Figure 2.9, panel a) compare with the non-CSC population, as expected, JAM-A
**Figure 2.8. JAM-A is a direct target of miR-145.** Luciferase assays confirm that the 3'UTR region is a target of miR-145 in two specimens evaluated. ** p<0.01, *** p<0.001 as assessed by one-way ANOVA.
Figure 2.9. miR-145 is inversely related to stem cell marker expression. An additional specimen confirms reciprocal expression of miR-145 and JAM-A in CSCs (a). A similar difference is observed when bulk tumor cells are compared with CSCs in both specimens analyzed (b). ** p<0.01, *** p<0.001 as assessed by one-way ANOVA.
expression followed the opposite pattern (between 2 and 4-fold increased expression in the CSCs). As a control, levels of SOX2 and OLIG2 were measured, and results confirmed between a 2- and 8-fold increase in expression of these CSC markers in CSCs compared with non-CSCs. Similar expression differences were observed when CSCs were compared with unenriched cells from a freshly dissociated tumor (Figure 2.9, panel b).

2.3.3 miR-145 introduction downregulates JAM-A and compromises self-renewal

I next sought to evaluate the effect of miR-145 gain of function in CSCs. I confirmed that JAM-A mRNA and protein levels were downregulated in CSCs after miR-145 introduction compared with cells containing NT mimics (Figure 2.10, panels a and b and Figure 2.13, panel a). Paralleling the results of JAM-A overexpression, the decrease in JAM-A was associated with a decrease in p-AKT and SOX2 (Figure 2.10, panel b and Figure 2.13, panel a). I also analyzed whether the repression of these proteins had a functional consequence in CSCs using in vitro self-renewal assays. Indeed, introduction of miR-145 decreased the stem cell frequencies as well as the number of spheres formed (Figure 2.11 and Figure 2.13, panel b) compared with the NT mimics group. Moreover, miR-145 has been previously reported to regulate pluripotency factors (Xu et al., 2009). I found that upon introduction of miR-145 into CSCs, the levels of SOX2, OCT4, and NANOG were reduced (Figure 2.12), suggesting a role for miR-145 in
**Figure 2.10. miR-145 introduction downregulates JAM-A.** Restoration of miR-145 in CSCs reduces mRNA (a) and protein levels (b) of JAM-A together with protein levels of SOX2 and p-AKT (b). **p<0.01, ***p<0.001 as assessed by one-way ANOVA.
Figure 2.11. **miR-145 introduction compromises self-renewal.** Restoration of miR-145 in CSCs compromises self-renewal in CSCs, as depicted by decreased stem cell frequencies (c) in the cells that received miR-145 mimics compared with the NT mimics.
Figure 2.12. miR-145 introduction downregulates stem cell genes. Restoration of miR-145 in CSCs decreases SOX2, NANOG, and OCT4 mRNA levels (d). ** p<0.01, *** p<0.001 as assessed by one-way ANOVA.
Figure 2.13. miR-145 downregulates JAM-A expression and compromises sphere formation. T387 CSCs corroborate a decrease in JAM-A together with p-AKT and SOX2 at the protein level upon miR-145 introduction (a). Functionally, this also decreases the number of cells formed per well at the 10 cells/well seeding density (b). *** p<0.001 as assessed by one-way ANOVA.
regulating core self-renewal genes. In addition, I also assessed whether SOX2 levels affected the expression levels of miR-145, as previously hypothesized (Fang et al., 2011), by knocking down Sox2 using two independent shRNA constructs (Figure 2.14, panel a). I measured miR-145 expression levels after Sox2 knockdown and found a reduction, not an induction as previously predicted, in both KD groups compared with the control group (Figure 2.14, panel b). Taken together, these data suggest that lower levels of miR-145 are critical for JAM-A expression and are not dependent on SOX2 levels, and that miR-145 regulates CSC self-renewal.

2.3.4 JAM-A overexpression rescues CSC self-renewal after miR-145 introduction

To confirm that the effects on self-renewal induced by miR-145 were dependent on JAM-A, I analyzed the behavior of JAM-A-overexpressing cells after treatment with NT and miR-145 mimics. I observed that JAM-A protein levels were reduced upon miR-145 introduction but were rescued by JAM-A overexpression (Figure 2.15, panels a and b). Importantly, SOX2 protein levels correlated with JAM-A expression; thus, JAM-A overexpression also rescued SOX2 in both specimens analyzed (Figure 2.15, panels a and b). Functionally, as assessed by in vitro limiting dilution analysis, I also demonstrated that the miR-145-induced decrease in self-renewal was rescued by JAM-A overexpression (Figures 2.15, panels c and d). Additionally, a reduction in the levels of miR-145 was observed in CSCs
Figure 2.14. Sox2 knockdown does not induce miR-145 levels. Two independent shRNA constructs decrease SOX2 expression at the protein level (a) but fail to induce miR-145 levels (b) as previously reported in the literature. ** p<0.01 as assessed by one-way ANOVA.
Figure 2.15. JAM-A overexpression rescues CSC self-renewal after miR-145 introduction. miR-145 introduction into CSCs overexpressing JAM-A does not decrease JAM-A or SOX2 protein levels (a and b). Limiting dilution analysis of CSCs containing miR-145 mimics shows a lower stem cell frequency compared with NT mimics and both JAM-A-overexpressing groups (c and d).
treated with JAM-A vector compared with control vector (Figure 2.16, panel a), suggesting a double feedback mechanism. JAM-A overexpression led to an increase in p-AKT, which is key for CSC maintenance (Bleau et al., 2009; Eyler et al., 2008) and has been shown to regulate miR-145 (Sachdeva et al., 2012). Thus, I treated CSCs with a PI3K inhibitor (LY294002) or an AKT inhibitor (MK226) and measured the levels of miR-145. Indeed, miR-145 levels were 2-3-fold higher in inhibitor-treated cells compared with the DMSO group (Figure 2.16, panel b), confirming a two-way regulation system in this pathway. The data described above lead to a regulatory axis whereby: i) JAM-A is preferentially expressed in CSCs, and miR-145 (which regulates JAM-A) is downregulated in the same population; ii) overexpression of JAM-A is associated with elevated p-AKT levels and decreased miR-145; and iii) miR-145 also regulates self-renewal genes such as SOX2, OCT4, and NANOG (Figure 2.17). In CSCs, increased levels of JAM-A can block the normal inhibition of stem cell genes by miR-145, thereby indirectly generating an increase in self-renewal and promoting malignancy and tumorigenesis.

2.3.5 Lower miR-145 expression is associated with decreased patient survival

To interrogate the clinical relevance of miR-145 and JAM-A in patient prognosis, I evaluated The Cancer Genome Atlas (TCGA) dataset (Cancer Genome Atlas Research, 2008) and found that miR-145 levels were predictive of overall GBM patient survival, with patients with lower levels of miR-145 having significantly
Figure 2.16. JAM-A/ miR-145 double feedback mechanism. miR-145 expression levels are downregulated with JAM-A overexpression (a) and upregulated in response to both PI3K (LY) and AKT (MK226) inhibitors (b). * p<0.05, ** p<0.01 as assessed by one-way ANOVA.
Figure 2.17. JAM-A/ miR-145 double feedback mechanism working model. Schematic of the proposed mechanism, where miR-145 regulates the levels of JAM-A and self-renewal genes, while JAM-A activates the AKT pathway and regulates miR-145 expression, driving an increase in self-renewal.
Figure 2.18. Low miR-145 levels are associated with poor patient prognosis. Survival curves in all GBM patients in TCGA dataset show that low miR-145 levels are informative for poorer patient survival (a), and a robust difference is also seen when miR-145 is combined with JAM-A (b) and NANOG (c) but not with SOX2 (d). ** p<0.01 as assayed by one-way ANOVA compared with the mesenchymal subtype. The log-rank p-value for significance between groups is shown on each graph.
poorer prognosis (Figure 2.18, panel a). When miR-145 levels were combined with JAM-A expression from the same patients, the difference in survival was enhanced (Figure 2.18, panel b), with high JAM-A/low miR-145 correlating with much poorer prognosis. This difference was also observed when miR-145 was combined with NANOG (Figure 2.18, panel c) but not with SOX2 (Figure 2.18, panel d). The median survival was the lowest when low miR-145 was combined with JAM-A (Table 2.1, top), and in general, median survival was lower in the groups with low miR-145 levels. This further demonstrates that JAM-A and stem cell genes combined with miR-145 levels can predict survival in GBM patient datasets. Similarly, the levels of miR-145 in the different subtypes of GBM in the TCGA dataset were evaluated and I found that the proneural subtype had the lowest expression (Figure 2.19, panel a). Interestingly, a similar analysis evaluating the expression of JAM-A also showed lower expression in the proneural group (Figure 2.19, panel a). This suggests there could be additional components in the JAM-A/miR-145 regulatory axis that require further study. Finally, I evaluated the survival curves of the different subtypes for low and high miR-145 expression and observed a significant difference in both the classical and proneural subtypes but not in the mesenchymal subtype (Figure 2.19, panel b). When patients with low miR-145 were combined with high JAM-A, SOX2, and OCT4 in these subtypes, I observed that the median survival was consistently lower than their counterpart groups (Table 2.1, bottom). Although only one group comparison reached significance (low miR-145/high SOX2 in the mesenchymal subtype), I noted that the lowest median survival was found in the proneural
Figure 2.19. Differential expression of JAM-A. The proneural subgroup had the lower expression levels of both JAM-A (a) and miR-145 (b). Patients in the classical, proneural, and mesenchymal molecular subtypes were analyzed for low and high miR-145 expression, showing that miR-145 is informative in the classical and proneural subtypes (b). * p<0.05, ** p<0.01, *** p<0.001 as assessed by one-way ANOVA; red: compared with the mesenchymal subtype, blue: compared with the classical subtype.
Table 2.1. Summary of median survival in GBM molecular subtypes using the TCGA dataset. Summary table of the p-values generated when log-rank statistical analyses were used to compare low vs. high miR-145 levels in all TCGA and different GBM subtypes and the combination of miR-145 with stem cells genes in all TCGA (top); combinations in each of the subtypes and the corresponding values are also summarized (bottom).

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subtype, implying that these genes could be more informative for proneural GBM patient survival.

2.4 Discussion

JAM-A has recently been demonstrated to be essential for CSC maintenance and dispensable for NPC function (Lathia et al., 2014), thereby representing a potential therapeutic target. The interactions presented in this dissertation corroborate the importance of JAM-A, as its ectopic expression is capable of increasing stem cell frequency and proliferation. This effect, however, was differential in the specimens tested and could be due to differences in molecular subtype. Future studies would benefit from expanding this work to additional specimens of different genomic backgrounds to how broadly applicable this JAM-A/miR-145 regulatory axis is in CSC maintenance.

To uncover the mechanism by which self-renewal is coordinated by adhesion and integrated into a larger signaling network, I interrogated microRNAs and found evidence for a double feedback mechanism between JAM-A and miR-145, where the latter binds directly to the 3'UTR region of the JAM-A message, attenuating self-renewal. This miR-145 signaling system extends beyond JAM-A to core pluripotency factors (SOX2, OCT4, and NANOG), which were also downregulated upon miR-145 mimic introduction into CSCs. These data are consistent with previous reports that described miR-145 as a regulator of SOX2, OCT4, and KLF4 in human embryonic stem cells (Xu et al., 2009), miR-145 as a
tumor suppressor in GBM via suppression of NEDD9 (Speranza et al., 2012), and a critical role for miR-145 in cell migration and self-renewal (Lee et al., 2013). I also described an association between miR-145 levels and patient survival based on data from The Cancer Genome Atlas. This showed that patients with lower levels of miR-145 (and in combination with high JAM-A levels) had a lower median survival than patients with high miR-145 levels. Despite the fact that this association was statistically significant, it should be tested in other independent cohorts and evaluated as a prognostic factor for patient survival. Similarly, future studies should analyze the ability of JAM-A to rescue the attenuated glioma-initiating capacity induced by ectopic expression of miR-145.

The implications of this signaling network extend beyond GBM, as miR-145 regulates JAM-A expression in breast cancer (Gotte et al., 2010), and more recently, the expression of JAM-A was associated with decreased malignancy and invasiveness in malignant mesothelioma (Cioce et al., 2014), bladder cancer (Kou et al., 2014), and lung adenocarcinoma (Hu et al., 2014). In addition to the link between JAM-A/miR-145 and pluripotency factors, JAM-A/miR-145 signals through the AKT pathway, a key CSC maintenance signaling node (Bleau et al., 2009; Eyler et al., 2008). The regulation of miR-145 by AKT may occur via activation of C/EBP-β as previously described (Sachdeva et al., 2012). This interaction also likely explains why JAM-A overexpression is accompanied by activation of the AKT pathway and decreased miR-145 expression. However, miR-145 reduction may also be due to JAM-A activation via an adjacent cell, and
this interaction represents a starting point for future inquiry as to how JAM-A interaction between multiple cell types within the tumor (CSCs, non-CSCs, endothelial cells, immune cells) drives self-renewal. Taken together, our data demonstrate that CSCs possess specific mechanisms to preserve cell adhesion molecules and self-renewal genes that include a miR-145/JAM-A axis that drives CSC maintenance.
Chapter 3: TLR4 suppresses CSC

maintenance
3.1 Introduction

In parallel to normal development, a self-renewing, tumorigenic CSC population has been identified in many advanced cancers (Visvader and Lindeman, 2012), including GBM, the most common malignant primary brain tumor (Ostrom et al., 2015). CSCs drive tumor growth (Singh et al., 2004) and therapeutic resistance (Bao et al., 2006; Chen et al., 2012) and are not solely governed by intrinsic programs but also influenced the tumor microenvironment that is essential in maintaining the balance between self-renewal and differentiation (Lathia et al., 2015). Among the extrinsic stimuli, there are some features that are byproducts of rapid and uncontrolled proliferation including hypoxia, acidic stress, and necrosis that are not favorable to cell survival; yet CSCs have adapted mechanisms to self-renew, despite these inhospitable stimuli (Hjelmeland et al., 2011; Li et al., 2009). However, a direct link between these response mechanisms and the core self-renewal signaling circuitry has yet to be elucidated.

The innate immune system recognizes both damage and pathogen associated molecular patterns and has evolved as an early response mechanism (Bianchi, 2007). In the tumor microenvironment, the existence of damage signaling could impact the phenotype and activation status of tumor cells as well as infiltrating immune cells. Responses to these ligands are mediated by distinct classes of receptors that sense and initiate pro-inflammatory signaling cascades. Among these, the toll-like receptor (TLR) family activates inflammatory response
pathways and is essential for the recruitment of effector immune cells (O'Neill et al., 2013). Receptor diversity accommodates a wide spectrum of ligands and accounts for an equally diverse set of responses from members of the TLR family. In the context of cancer, TLRs have both pro- and anti-tumorigenic roles that are tumor- and cell-type specific (Pradere et al., 2014). The anti-tumorigenic roles are generally due to induction of anti-tumor immunity via activation of dendritic cells (Pradere et al., 2014). The pro-tumorigenic roles are mediated by tumor-associated macrophages, dendritic cells, and endothelial cells directly along with TLR-mediated cytokine production (Elinav et al., 2013). In multiple tumor types, including hepatocellular carcinoma, colorectal, prostate, and bladder, TLRs appear to function more as pro-tumorigenic (Cheah et al., 2015; Grimm et al., 2010; Zhao et al., 2015b) and the precise outcome of TLR signaling in tumor cells, including CSCs, is an ongoing area of investigation.

There is accumulating evidence that TLRs may be important for CSC maintenance. In hepatocellular carcinoma, TLR4 directly interacts with the core self-renewal gene NANOG to drive CSC maintenance (Chen et al., 2013). In GBM, it has recently been demonstrated that self-renewal is mediated by TLR9 (Herrmann et al., 2014). Similar pro-proliferative roles have been described for TLR4 in embryonic stem cells and mammary progenitor cells (Lee et al., 2009); however anti-proliferative roles have also been described for TLR2, TLR3, and TLR4 in embryonic (Lathia et al., 2008; Okun et al., 2010) and adult neural progenitor cells (Rolls et al., 2007). These findings substantiate that distinct TLRs
can modulate the proliferation of non-immune cells in a positive or negative manner. As tumor cells reside within an inhospitable environment, we became interested in the adaptive mechanisms that CSCs possess to ignore these damage associated signals. The innate immune machinery, regulated by TLRs, are critical regulators of damage signaling and we therefore interrogated GBM CSCs versus their non-stem cell (non-CSC) counterparts for the expression of TLRs and how these receptors impacted self-renewal. We found that TLR4 has the capacity to be tumor suppressive and CSCs lose TLR4 expression to thrive in a hostile environment and avoid activation of inflammatory pathways. Taken together, our findings demonstrate that CSCs have reduced innate immune signaling activation, that when activated, directly suppresses CSC maintenance.

3.2 Materials and Methods

3.2.1 *In vitro* functional analysis

In order to functionally study CSCs *in vitro* two experimental approaches have been utilized. The first test measures proliferation of cells under different conditions and after genetic modifications. In this manner, cell growth can be assessed which represents one of the key defining concepts of CSCs. The second test is an *in vitro* surrogate of self-renewal. By plating low number of cells and measuring their capacity to grow into spheres we can assess their true stem cell potential. Cell proliferation experiments were conducted by plating cells of interest at a density of 1000 cells/well in a 96-well plate in triplicate. Cell number
was measured every other day and normalized to the initial reading at Day 0 using the CellTiter-Glo assay kit (Promega, Madison, WI, USA). For tumorsphere formation experiments, cells were FACS sorted (FACS Aria II) into 96-well plates at a density of 1, 5, 10 and 20 live cells per well (24 wells for each density). Cells were maintained for 10 days before sphere formation was evaluated. Spheres larger than 10 cells in diameter were considered for analysis. Reported numbers represent either number of cells per well or stem cell frequency calculated using the Walter and Eliza Hall Institute Bioinformatics Division ELDA analyzer (http://bioinf.wehi.edu.au/software/elda/).

### 3.2.2 Toll-like receptor signaling modulation

The following TLR ligands and inhibitors were obtained from InvivoGen (San Diego, CA, USA): CpG (2 µM), Pam3C (300 ng/mL), PolyI:C (10 µg/mL), CLI-095 (1 µM), and BX795 (100 nM). LPS (500 ng/mL) was obtained from Sigma. HMGB1 (1 µg/mL) was obtained from (GenScript, Piscataway, NJ, USA). Concentrations used in the experiments presented are specified in brackets.

### 3.2.3 Immunoblotting analysis

Cell populations were lysed using RIPA lysis buffer (containing PMSF, protease inhibitor cocktail, and sodium orthovanadate; Santa Cruz Biotechnology, Dallas, TX, USA), and protein concentrations were calculated using a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). After denaturation with Laemmli buffer (BioRad Laboratories, Hercules, CA, USA), 10 µg of total protein was
loaded on 10% polyacrylamide SDS-PAGE gels, transferred to polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and probed using the following antibodies: TLR4 (Santa Cruz Biotechnology, 1:1000), SOX2 (R&D Systems, 1:1000), RBBP5 (Cell Signaling, Danvers, MA, USA, 1:2000), GFAP (Invitrogen, 1:1000), TBK1 (Cell Signaling, 1:500), and phospho-TBK1 (Cell Signaling, 1:500); β-Actin (Santa Cruz Biotechnology, 1:2000) was used as a loading control. Species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies were used for detection (Invitrogen, 1:5000). Membranes were developed using ECL-2 reagent (Pierce Biotechnology).

3.2.4 qRT-PCR

RNA from cells of interest was extracted using TRIzol (Life Technologies), and cDNA was synthesized using the Superscript III kit (Invitrogen). qPCR reactions were performed using an ABI 7900HT system using SYBR-Green Mastermix (SA Biosciences, Valencia, CA, USA). For qPCR analysis, the threshold cycle (CT) values for each gene were normalized to expression levels of β-Actin. Dissociation curves were evaluated for primer fidelity, and only threshold cycles below 35 cycles were reported. The primers (Integrated DNA Technologies) used can be found in the Supplemental Materials and Methods. A list of the primers used in these experiments can be found in the Appendix I section.

3.2.5 Flow cytometry
Bulk tumor cells dissociated the day before were analyzed for cell surface expression using the following fluorophore-conjugated antibodies: TLR4-PE (Santa Cruz, 1:50), CD133-FITC (Miltenyi Biotech 1:50), and CD49f-APC (Miltenyi Biotech, 1:50). Cells were incubated with these antibodies for 30 minutes at room temperature, washed with PBS, and analyzed using BD LSRFORTESSA with DAPI (Sigma, 1:10000) as a control for live cells.

### 3.2.6 Patient database bioinformatics

Gene expression data was obtained from different databases using GlioVis ([http://gliovis.bioinfo.cnio.es](http://gliovis.bioinfo.cnio.es)) for patients with GBM. Patients were divided into low (mean – on standard deviation) and high (mean + one standard deviation) expressing groups for each TLR. The remaining patients (within one standard deviation of the mean) composed the median group. Survival analysis was done utilizing Kaplan-Meier statistical test comparing the groups via log-rank test. Similarly, databases were analyzed to study the correlation between the expression of RBBP5 and SOX2 using Pearson coefficient analysis.

### 3.2.7 Lentiviral shRNA and overexpression preparation

Lentiviral constructs were prepared according to modified protocols from Tronolab ([http://tronolab.epfl.ch](http://tronolab.epfl.ch)). In short, using calcium phosphate precipitation, 293FT cells were co-transfected with the packaging vectors psPAX2 and pMD2.G (Addgene, Cambridge, MA, USA) and lentiviral vectors directing the expression of i) MISSION shRNA (Sigma) specific to RBBP5: TRCN0000353567
(KD1) and TRCN0000369176 (KD2)) or a non-targeting control (NT) shRNA (SHC002) and ii) overexpression of TLR4: accession number BC117422 (LV335816); Applied Biological Materials, Richmond, Canada), overexpression of RBBP5: accession number BC053856 (LV283511) or control vector (LV590) to produce virus. Media on the 293FT cell cultures were changed 18 hours after transfection, and viral supernatants were collected 12, 24, and 36 hours later and concentrated using polyethylene glycol precipitation for immediate use or stored at -80°C for future use.

### 3.2.8 Luciferase promoter reporters

Cells containing NT or RBBP5 KD were used for transfection with luciferase constructs. A total of 500,000 cells per condition were plated in 6-well plates pretreated with Geltrex (Life Technologies), which was used as an adherence substrate. The following day, cells were transfected with each of the promoter reporters (Genecopoeia) using Lipofectamine 2000 (Life Technologies) with 2 µg of DNA per reaction in Opti-Mem (Lerner Research Institute Media Core) medium. After 6 hours of incubation at 37°C, Opti-Mem was replaced with complete Neurobasal. 48 hours later, media was collected and secreted *Gaussia* Luciferase was analyzed using Secrete-Pair *Gaussia* Luciferase (Genecopoeia) per the manufacturer’s instructions.

### 3.2.9 *In vivo* intracranial injections
For *in vivo* tumor formation, NT or RBBP5 KD vector containing live CSCs were transplanted into the frontal lobe of NOD scid gamma (NSG) mice at 1000 cells per mouse (n=7 for KD and n=6 for control). Mice were monitored daily and sacrificed upon the development of neurological signs.

### 3.2.10 Statistical analysis

Reported values are mean values +/- standard error of the mean from studies performed at least in triplicate. Unless otherwise stated, one-way ANOVA was used to calculate statistical significance, with p-values detailed in the text and figure legends.

### 3.3 Results

#### 3.3.1 Differential response to innate immune stimulation and lower expression of TLR4 in CSCs

Several efforts have been directed in recent years to modulate the immune system, which is a critical component of the tumor microenvironment in GBM. One of these approaches is the use of compounds aimed at boosting immune cells such as T cells and macrophages. Toll-like receptors recognize foreign patterns on invading microbes to induce inflammatory pathways. Ligands for these receptors can be used to stimulate immune cells yet their effect on malignant tumorigenic cells has not been thoroughly explored. Thus, I investigated the effect of several TLR ligands on the proliferation of both CSCs
Figure 3.1. Differential response to innate immune stimulation. Proliferation levels after 7 days of incubation with TLR ligands in CSCs (blue) and non-CSCs (black) as compared with internal controls to which these were normalized ** p<0.01 as assayed by one-way ANOVA.
and non-CSCs (Figure 3). In all the specimens analyzed, ligands that stimulate TLR2 (Pam3C), TLR3 (polyinosinic:polycytidylic acid, PolyI:C), and TLR9 (oligodeoxynucleotides, CpG) showed no statistically significant effect on either cell population. However, TLR4 agonist lipopolysaccharide (LPS) showed a differential effect on tumor cells where CSCs seemed to proliferate normally while non-CSCs were severely affected (Figure 3.1). In order to confirm this effect was TLR4-specific, I used a signaling inhibitor (CLI-095) and saw that we could abrogate the decrease in proliferation induced by TLR4 stimulation by adding CLI-095 (Figure 3.2). In the brain context, normal and tumor cells will rarely be exposed to microbial components such as TLR ligands however there are endogenous proteins that can also serve as activators for this family of receptors. During necrosis and other injury/stress related cellular processes, proteins that are usually found inside cells can be released and act as damage associated molecular patterns (DAMPs). These can be recognized by TLRs in order to activate inflammatory pathways and/or recruit immune cells from the periphery. I therefore confirmed our findings stimulating cells with high mobility group box 1 (HMGB1), a TLR4 and TLR2 endogenous ligand (Figure 3.1). As expected, only the non-CSC cellular compartment was affected showing a decrease in proliferation as compared with the CSCs. Again, treating cells with a TLR4 signaling inhibitor (CLI-095) blocks the decrease in proliferation induced by HMGB1 (Figure 3.2). Additionally, TLR ligands were used to stimulate bulk tumor cells plated for limiting dilution analysis. Stem cell frequencies were similar to that
Figure 3.2. Signaling specificity of TLR4 signaling. The addition of TLR4 signaling inhibitor (CLI-095) abrogates reduction in proliferation induced by both LPS and HMGB1; normalized to control group. ** p<0.01 as assayed by one-way ANOVA.
Table 3.1. TLR ligands do not affect self-renewal of bulk tumor cells. Bulk tumor cells of two specimens were plated in a limiting dilution manner in the presence of several TLR ligands. Stem cell frequencies and p-values are indicated for each treatment group.

### 4121 xenograft

<table>
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<td>control</td>
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</tr>
<tr>
<td>LPS</td>
<td>1 in 3.8 (2.7-5.3)</td>
<td>0.06</td>
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<tr>
<td>Poly (I:C)</td>
<td>1 in 2.4 (1.8-3.3)</td>
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<td>Pam3C</td>
<td>1 in 7.4 (5.2-10.7)</td>
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<tr>
<td>CpG</td>
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### 3832 xenograft

<table>
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<tbody>
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<tr>
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<td>0.46</td>
</tr>
<tr>
<td>CpG</td>
<td>1 in 2.5 (1.9-3.5)</td>
<td>0.46</td>
</tr>
</tbody>
</table>
of the control group in almost all of the cases except when Pam3C showed an increase in stemness in one of the xenografts tested (Table 3.1).

Similarly, I evaluated the expression of all human TLRs in CSCs and non-CSCs. Several significant differences were noted where TLR2 and TLR9 were highly expressed while TLR3, TLR4, and TLR8 were lowly expressed in the CSCs compared with non-CSCs (Figure 3.3). I therefore hypothesized that CSCs stimulated with TLR4 ligands (LPS and HMGB1) are not affected because they have lower TLR4 expression. These observations were confirmed by analyzing additional specimens at both the mRNA (Figure 3.4, panel a) and protein (Figure 3.4, panel b) levels. Consistently, CSCs showed minor levels of TLR4 expression compared with the non-CSC population. Interestingly, lower levels of TLR4 in patients are associated with a decreased median survival (Figure 3.4, panel c). Similar analyses performed for TLR2, TLR3, TLR8, and TLR9 did not show these receptors were informative of patient survival (Figure 3.5, panel a). In addition, other databases confirm our findings with TLR4 (Figure 3.5, panel b). These data show CSCs are not as responsive as non-CSCs to damage signals that stimulate TLR4 and that the expression of this receptor is informative of median survival in GBM patients.

3.3.2 TLR4 is associated with the stem cell state

In order to study if TLR4 expression has functional implications I decided to evaluate stem cell characteristics of cells expressing the receptor. When the
Figure 3.3. Differential expression of TLR family in tumor cell populations. mRNA expression levels of all known human TLRs were evaluated in CSCs and non-CSCs; data normalized to non-CSC group. * p<0.05, ** p<0.01, *** p<0.001 as assayed by one-way ANOVA.
Figure 3.4. CSCs express low TLR4 levels. mRNA (a) and protein (b) levels of TLR4 were analyzed in CSCs and non-CSCs using qRTPCR and western blot, respectively. (c) Kaplan-Meier survival plot shows TLR4 low levels are associated with poorer prognosis in the Phillips dataset. The log-rank p-value for significance between groups is shown next to the survival curve. *** p<0.001 as assayed by one-way ANOVA.
Figure 3.5. Additional TLRs bioinformatics. (a) Kaplan-Meier survival plots show TLR2, TLR3, TLR8, and TLR9 are not informative of patient survival. (b) Additional databases confirm TLR4 low levels are associated with poorer patient prognosis. The log-rank p-value for significance between groups is shown next to the survival curve.
Figure 3.6. TLR4 is associated with the stem cell state. (a) Percentage of cells expressing TLR4 before (left panel) and after (right panel) enrichment for CSC using CD133 was determined using flow cytometry. (b) Bulk tumors derived from patient specimens were plated in a limited dilution manner based on TLR4 levels and the number of wells containing spheres was counted in order to generate stem cell frequencies.
Figure 3.7. TLR4 is associated with the stem cell differentiation. CSCs were differentiated in DMEM containing 10% FBS and protein expression levels of TLR4, SOX2, GFAP, and Actin were assessed at the indicated time points.
Figure 3.8. TLR4 is associated with stem cell state. (a) Additional specimen showing TLR4 low levels have higher stem cell frequencies compared with high TLR4 expressing cells from the same tumor. (b) Additional specimen showing TLR4 levels increase after differentiation together with a decrease in stem cell marker SOX2 and an increase in differentiation marker GFAP at the protein level.
expression of TLR4 in the bulk of a xenograft was analyzed, 43% of the cells were TLR4 positive, however after enrichment with a CSC marker, CD133, less than 1% were positive (Figure 3.6, panel a). Moreover, TLR4 levels of expression were used to plate cells in limiting dilution analysis experiments. In several specimens, TLR4 low expressing cells had higher stem cell frequencies than cells expressing high TLR4 levels (Figure 3.6, panel b and Figure 3.8, panel a). Similarly, I studied the pattern of TLR4 expression when stemness conditions of culture were modulated. When I placed CSCs in differentiation media (containing 10% FBS) I saw a gradual increase in the expression of TLR4 together with a concomitant decrease in the expression of SOX2, a stem cell marker (Figure 3.7 and Figure 3.8, panel b). GFAP was used as a marker of astrocytic lineage differentiation. These data show TLR4 is not only downregulated in the CSCs but also has a negative correlation with stemness, where modulation of the latter can affect expression of the former.

**3.3.3 TLR4 overexpression is anti-proliferative and reduces CSC maintenance**

Conversely, I evaluated if the modulation of TLR4 expression in CSCs translated to a functional level. Using lentiviral constructs I was able to stably express TLR4 at both the protein (Figure 3.9, panel a) and mRNA (Figure 3.9, panel b) levels. Remarkably, forced expression of TLR4 induced a decreased in proliferation even in the absence of a TLR4 ligand (Figure 3.10, panel a). When LPS was added a further decrease was observed in proliferation of the TLR4
Figure 3.9. TLR4 overexpression reduces CSC maintenance. Increased levels of TLR4 were confirmed at both protein (a) and mRNA (b). *** p<0.001 as assayed by one-way ANOVA.
Figure 3.10. TLR4 overexpression is anti-proliferative and reduces CSC maintenance. (a) Proliferation was measured in several specimens after overexpression of TLR4 using a lentiviral vector. Levels were normalized to a control vector. (b) LPS was used to stimulate cells after TLR4 overexpression and proliferation was assessed after 7 days. (c) Limited dilution analysis was utilized to estimate stem cell frequencies in CSCs after transfection with control or TLR4 overexpression vectors. ** p<0.01 as assayed by one-way ANOVA.
overexpressing cells (Figure 3.10, panel b) but not in the control group (data not shown). These data show that TLR4 modulation alone is able to induce a decrease in proliferation and adding a TLR4 ligand accentuates this effect. Likewise, TLR4 overexpressing cells show lower stem cell frequencies compared with cells expressing a control vector (Figure 3.10, panel c). This dramatic effect (1 in 35 and 1 in 27 to 1 in 120 and 1 in 71, respectively) further supports the decrease in proliferation and evidences the fact that activation of innate immune signaling can be detrimental for cancer cells. Previous results showed that TLR4 levels increased when CSCs were differentiated and here I saw that forced TLR4 expression resulted in a decrease in SOX2 levels (Figure 3.9, panel a). This observation was critical for the studies described in the next sections.

3.3.4 TLR4 overexpression compromises stemness and validation via transcription factor screen

In order to elucidate the mechanism by which TLR4 is able to regulate proliferative and self-renewal pathways, I decided to assess the expression of stem cell genes such as SOX2, OCT4, and NANOG (Figure 3.11). In both specimens analyzed it is clear that overexpression of TLR4 induces a decrease in the expression of these genes. To further explore this interaction a bioinformatics approach was used, outlined in Figure 3.12, panel a. First I analyzed the promoter region of all three stem cell genes using UCSC genome browser and compiled a list of common transcription factors that can bind to all three regions. The expression of these was explored in a RNA-seq dataset
Figure 3.11. TLR4 overexpression reduces stem cell gene expression. Stem cell gene expression was measured at the mRNA level after TLR4 overexpression in CSCs; normalized to control vector. ** p<0.01, ***p<0.001 as assayed by one-way ANOVA.
Figure 3.12. Validation via transcription factor screen. (a) Workflow of bioinformatics approach to identify downstream effectors of TLR4. (b) After transcription factors were validated in an RNA-seq dataset, their mRNA expression levels were assessed in the context of TLR overexpression in CSCs; normalized to control vector. (c) Protein levels of RBBP5 were analyzed using western blot in both CSCs and non-CSCs. * p<0.05, ** p<0.01, *** p<0.001 as assayed by one-way ANOVA.
### Table 3.2. Transcription factor expression in RNA-seq dataset.

Transcription factors (TFs) found to bind to the promoter regions of stem cell genes were analyzed and ratios were created by comparing the expression in tumor promoting cells (TPCs) with differentiated glioma cells (DGCs). TFs factors with ratios in red were selected for subsequent step. TFs without expression values were absent in the RNA-seq dataset.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>TPCs/DGCs average (Suva et al)</th>
<th>TLR4 / control vectors</th>
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<tr>
<td>BACH1</td>
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<tr>
<td>EZH2</td>
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<tr>
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<td>CTBP2</td>
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<tr>
<td>TAF7</td>
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</tr>
<tr>
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(Suva et al., 2014), which includes RNA expression in tumor propagating cells (TPCs) compared with differentiated glioblastoma cells (DGCs); the ratios of expression of the available genes are shown in Table 3.2. In only considered transcription factors that were 1.5-fold differentially expressed and evaluated their expression in the context of TLR4 overexpression (Figure 3.12, panel b). Notably, some of the differentially expressed genes in the RNA-seq dataset do not seem to be affected by the levels of TLR4 (REST, SOX5, TEAD4, POU3F2, and MYC). JUN, a known downstream effector of TLR signaling, is downregulated when TLR4 is overexpressed. However, other subunits of this transcription factor (JUNB, JUND, and FOS (not shown)) did not show the same trend and some even followed an opposite trend. This is not surprising based on the differential role of different activating protein (AP-1) subunits during inflammation and cancer (Hess et al., 2004). Yet, these interactions are interesting and worth examining in future studies. The most dramatic effect was seen with retinoblastoma binding protein 5 (RBBP5) where overexpression of TLR4 resulted in a 60% decrease in RBBP5 expression (Figure 3.12, panel b). Other than binding retinoblastoma as the name suggests, this protein is part of a core complex that interacts with SET1 family members to tri-methylate lysine 4 in histone 3 (Ernst and Vakoc, 2012). This epigenetic mark in DNA is considered to be a mark for active transcription. Of note, when I analyzed the expression of RBBP5 in both CSCs and non-CSCs I observed that its expression was higher in the CSC population suggesting it has an active role in the transcription machinery (Figure 3.12, panel c). In this context I evaluated the correlation
Figure 3.13. RBBP5 levels are correlated with SOX2. Correlation analysis (Pearson) was conducted in TCGA and Rembrandt databases showing a positive correlation of expression levels between RBBP5 and SOX2. Data was obtained using GlioVis.
between RBBP5 and SOX2, a known stem cell gene, using patient datasets in GlioVis. In all databases analyzed I saw a strong positive correlation between SOX2 and RBBP5 (Figure 3.13). These data identify RBBP5 as a downstream target of TLR4 and suggest a link between innate immune signaling and epigenetics that can regulate transcription machinery by methylation of lysine residues.

3.3.5 Targeting RBBP5 mimics TLR4 overexpression

In order to evaluate if RBBP5 was linked to the effect of TLR4 in stem cell genes I targeted RBBP5 using two independent short hairpin RNA (shRNA) lentiviral constructs in CSCs. I confirmed protein (Figure 3.14, panel a) and RNA (Figure 3.15) downregulation in two specimens. Importantly, targeting of RBBP5 resulted in decrease of SOX2 protein levels (Figure 3.14, panel a) and SOX2, OCT4, and NANOG mRNA levels (Figure 3.15). Additionally, I demonstrated transcription of these genes is suppressed using luciferase constructs controlled by their promoter region (Figure 3.14, panel b). This decrease in activity was translated functionally into a decrease in cell proliferation (Figure 3.16, panel a). Both specimens analyzed show a significant decrease in proliferation when RBBP5 is targeted with two shRNAs, knockdown 1 (KD1) and knockdown 2 (KD2), compared with a non-targeting (NT) control. The same effect was seen when cells treated with these KDs were evaluated for limiting dilution analysis (Figure 3.16, panel b). Stem cell frequencies were decreased from 1 in 2 to 1 in 13 (KD1) and 1 in 12 (KD2) for specimen T387 while 1 in 15 to 1 in 275 (KD1) and 1 in 133
Figure 3.14. Targeting RBBP5 validation in CSCs. (a) Validation of two shRNA constructs targeting RBBP5 at the protein level compared to non-targeting vector (NT). (b) Promoter reporters of the indicated genes controlling luciferase levels were used to measure transcription activation. * p<0.05, ** p<0.01, as assayed by one-way ANOVA.
Figure 3.15. Targeting RBBP5 decreases expression of stem cell genes. mRNA levels of the indicated were measured after transfection of two independent shRNA constructs; data normalized to non-targeting shRNA control. ** p<0.01, *** p<0.001 as assayed by one-way ANOVA.
Figure 3.16. Targeting RBBP5 mimics TLR4 overexpression in CSCs. (a) Proliferation levels were measured after knockdown of RBBP5 compared with NT. (b) Limiting dilution analysis of the effect of RBBP5 knockdown on two different specimens. ** p<0.01, *** p<0.001 as assayed by one-way ANOVA.
Figure 3.17. Targeting RBBP5 decreases tumor initiation in vivo. Kaplan-Meier survival plots after intracranial injection of 1000 T4121 of NT or RBBP5 knockdown cells. The log-rank p-value for significance between groups is shown next to the survival curve. * p<0.05, ** p<0.01, *** p<0.001 as assayed by one-way ANOVA.
(KD2) for specimen T4121. I further analyzed the targeting of RBBP5 in vivo for specimen T4121 and observed a decrease in tumor initiation capacity compared with the NT control (Figure 3.17). Mice injected intracranially with CSCs treated with RBBP5 shRNA did not show neurological deficiency symptoms (KD1 and KD2) after 75 days while NT treated CSCs formed tumors and mice implanted with these were sacrificed within 55 days of injection (median survival 43 days). These data highlight the importance of RBBP5 for the maintenance of CSCs and functional consequences when it is targeted. Notably, knockdown of RBBP5 mimics the effects seen when TLR4 is overexpressed in this cell population further suggesting RBBP5 can be a downstream effector of the activation of TLR4 signaling.

3.3.6 Ingenuity pathway analysis reveals TBK1 involvement

I decided to run an ingenuity pathway analysis (IPA) using TLR4, SOX2, and RBBP5 in order to elucidate other players in this signaling pathway (a simplified model is shown in Figure 3.18). The network shows an interaction with NANOG that was expected given our results with stem cell genes. It also shows nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), downstream of TLR4, which can also be regulated by SOX2 and is a common transcription factor activated by all TLRs. Interestingly, the network links TLR4 and RBBP5 via tank-binding kinase 1 (TBK1) that is known to be downstream of TLR4 in a myeloid differentiation primary response gene 88 (MyD88)-independent manner. To confirm these findings I assessed the effect of a TBK1 inhibitor, BX795, after
Ingenuity pathway analysis reveals TBK1 link with TLR4 and RBBP5.

Figure 3.18. Ingenuity pathway analysis reveals TBK1 link with TLR4 and RBBP5. Ingenuity pathway analysis (IPA) identifies TBK1 (orange) as a protein interacting partner of both TLR4 (blue) and RBBP5 (green), independent of NF-κB.
TLR4 stimulation. Indeed, treating non-CSCs with BX795 abrogated the decrease in proliferation induced by treatment with both LPS and HMGB1 (Figure 3.19). Similarly, the phosphorylation status of TBK1 upon stimulation was evaluated with these two TLR4 ligands. In both cases, the activation of TLR4 signaling led to increase in phosphorylation in TBK1 compared to untreated control (Figure 3.20, panel a). To study this pathway in CSCs I first overexpressed TLR4 and then analyzed the phosphorylation of TBK1. Clearly, forced expression of TLR4 induced an increase in the phosphorylation levels of TBK1 (Figure 3.20, panel b). I also wanted to link the activation of TBK1 with RBPP5 levels so non-CSCs were treated with LPS or LPS and BX795 (Figure 3.21). The mRNA levels of RBPP5 were decreased with LPS treatment and addition of BX795 was able to rescue RBPP5 levels to those of the untreated control. In a similar experiment, CSCs with or without forced expression of TLR4 were treated with the TBK1 inhibitor. As expected, TLR4 overexpression resulted in a decrease of RBPP5 and stem cell genes, SOX2, OCT4, and NANOG (Figure 3.20 panel c). Remarkably, treatment with BX795 in TLR4 overexpressing cells was able to rescue the mRNA levels of all the above-mentioned genes comparable to those of the control group. It is important to note that treatment of control cells with BX795 also lead to an increase in the expression of RBPP5 and stem cell genes raising the possibility of other signaling pathways that could be involved in regulating the stemness of CSCs. These data further confirm the effect of TLR4 in regulating stem cell pathways via RBPP5 and provide evidence
Figure 3.19. TBK1 inhibitor abrogates TLR4-induced decrease in proliferation. Proliferation levels of non-CSCs treated with LPS or HMGB1 in the presence or absence of TBK1 inhibitor (BX795); normalized to untreated control.
Figure 3.20. Increase in TBK1 phosphorylation levels after TLR4 stimulation and overexpression. (a) Protein levels of phospho-TBK1 are elevated after stimulation of non-CSCs with either LPS or HMGB1. (b) Phosphorylation of TBK1 is also increased in CSCs after TLR4 overexpression compared with control vector. (c) mRNA levels of indicated genes were assessed after TLR4 overexpressing or control cells were cultured in the presence or absence of TBK1 inhibitor (BX795); asterisk colors above bars represent statistical analysis compared with that specific experimental group. * p<0.05, ** p<0.01, *** p<0.001 as assayed by one-way ANOVA.
Figure 3.21. TLR4 stimulation modulates RBBP5 expression. mRNA levels of RBBP5 decreased upon stimulation with LPS. The effect was inhibited by addition of TBK1 inhibitor (BX795); data normalized to control group. * p<0.05 as assayed by one-way ANOVA.
pointing towards TBK1 as a key component of this signaling cascade (Figure 3.22).

3.4 Discussion

Our findings add to the increasing understanding of non-immune cell functions for TLRs in the brain that regulates proliferation, survival, and self-renewal in the context of development, neurodegeneration, and neoplasia. The brain has a distinct set of immune regulatory mechanism and diverse TLR expression on neurons, astrocytes, oligodendrocytes, and microglia contribute to normal brain function and homeostasis (Jack et al., 2005; Okun et al., 2009; van Noort and Bsibsi, 2009). It appears as if TLRs may be also regulating GBM growth and progression. TLR9 functions in a pro-tumorigenic manner to drive self-renewal (Herrmann et al., 2014) and versican-TLR2-MT1-MMP signaling axis has been linked to tumor invasion and expansion (Hu et al., 2015). However, TLR2 activation on dendritic cells leads to tumor regression and this is mediated via HMGB1, which is generated by stressors such as GBM standard of care irradiation and chemotherapy (Temozolomide) (Curtin et al., 2009). Along with more detailed studies to appreciate the cell-type specific role of individual TLRs during tumor growth, a more detailed analysis of endogenous ligands is required. It is surprising how little information is available as to the precise DAMPs present within the tumor microenvironment and how therapies may alter the DAMP signature. This is essential information that may help explain the alterations in
Figure 3.22. TLR4 suppresses cancer stem cell maintenance. Working model of the signaling axis where TLR4 activation leads to TBK1 phosphorylation that suppresses RBBP5, which regulates stem cell genes and is critical for CSC maintenance. In non-CSCs (left), TLR4 expression allows for the activation of TBK1 in the presence of appropriate TLR4 ligands and consequent inhibition of RBBP5 with concomitant decrease in stem cell gene activation. On the contrary, the lack of TLR4 expression in CSCs (right) suppresses the inhibition of RBBP5 and promotes activation of self-renewal programs with consequences in proliferation, growth, and tumor initiation. (Illustration by Amanda Mendehlson, Medical Art Department, CCF).
the immune system within GBM and reveal TLR-dependent interventions for follow-up studies.

Traditionally, TLR activation in non-tumorigenic cells has been associated with inflammation and the progression of tumor growth (Elinav et al., 2013). Recent evidence has also linked TLR activation in tumor cells with tumorigenesis in different oncogenic contexts such as hepatocellular carcinoma, colorectal, and prostate/bladder tumors. TLR4 in particular has been associated with driving hepatocellular carcinoma progression (Dapito et al., 2012) and interacts with the CSC signaling network via NANOG and STAT3 (Chen et al., 2013; Uthaya Kumar et al., 2015). In this manuscript, we describe the opposite role for TLR4 in GBM, suppressing tumor growth via directly attenuating the self-renewal circuitry. The tumor suppressive functions we observe with TLR4 in GBM are likely reflections of differences in the specialized immune environment within the brain as compared to that in the liver. From a signaling perspective, there are also differences in how TLR4 links to the self-renewal signaling network. We observed a repression of NANOG upon TLR4 activation while work in hepatocellular carcinoma observed an alcohol induced activation of NANOG, with coordinate activation of Akt and TGFbeta (Chen et al., 2013), both of which are major drivers of CSC maintenance in GBM (Anido et al., 2010; Bleau et al., 2009; Eyler et al., 2008). The divergence in signaling response could be due to ligand specificity or downstream pathway activation as TBK1 is a component of the MyD88-independent response and the activation of TLR4 may be working via a
MyD88-dependent mechanism in hepatocellular carcinoma. Further elucidating these differences in signaling pathways is an immediate priority as it may clarify pro- and anti-tumorigenic roles for TLR4. For example, it has recently been shown that TLR4 is necessary for epidermal growth factor receptor (EGFR) signaling in mammary epithelial cells via MyD88 (De et al., 2015) and given the importance of EGFR in GBM progression (Brennan et al., 2013) and CSC self-renewal (Mazzoleni et al., 2010), the activation MyD88-dependent or – independent pathways may provide diametrically opposite results. Furthermore, clarifying this difference will be vital information as TLRs modulation is being explored as an adjuvant immunotherapy for brain tumors (Deng et al., 2014).

Based on our findings, there may be therapeutic opportunities to target this new TLR4-TBK1-RBBP5-SOX2 signaling axis. While TLR4 suppress self-renewal, it remains a poor choice for therapeutic intervention as overexpressing a receptor is challenging to achieve in vivo. An alternative would be to increase p-TBK1 activity and this could be done via a specific activating agonists. Of note, targeting TBK1 is possible as inhibitors are currently under clinical evaluation for psoriasis and chronic obstructive pulmonary disease. Our data suggest that RBBP5 is a critical member of this axis and our pre-clinical genetic studies indicate that its reduction attenuates SOX2 expression, self-renewal, and tumor initiation. RBBP5 may be targeted by the development of specific small molecules or specific micro-RNAs that suppress its expression. While these
approaches would require substantial development, they may provide next-generation anti-CSC targeting strategies.

A key question that arises based on our findings is the link between innate immune activation and self-renewal. Conceptually, it may not be surprising that self-renewal is suppressed during damage response as other key cellular functions are also paused including proliferation. It could be advantageous to utilize cellular resources to respond to damage at the expense of generating additional progeny that would be immediately at risk for damage and possible apoptosis. However, in the context of cancer, genomic instability is a driver of tumor growth and GBM CSCs harbor enhanced DNA damage but compensate by possessing elevated DNA repair mechanisms (Bao et al., 2006). Unlike the situation for DNA damage, we find that innate immune activation is a potent suppressor of self-renewal, likely ensuring that CSC progeny are less susceptible to damage and apoptosis. This suppression is regulated by a signaling axis consisting of TLR4-TBK1-RBBP5-SOX2 that has not previously been reported and represents a unique mechanism by which innate immune activation can directly attenuate self-renewal. RBBP5 appears to be a key node in this signaling axis and essential for CSC maintenance, which has also previously not been reported. RBBP5 has been characterized to be elevated in GBM versus non-neoplastic brain tissue (Zhao et al., 2015a) and is considered a putative novel GBM oncogene based on exon-level expression profiling (Bralten et al., 2010). A major reported function of RBBP5 is the tri-methylation of lysine 4 in histone 3.
RBBP5 is a member for the WRAD complex and interacts with several SET1 family members, including MLL1 that has previously been demonstrated to be essential in CSC maintenance (Gallo et al., 2013; Heddleston et al., 2012) and interacts with HOX gene family members that are master regulators of embryonic development (Gallo et al., 2013). As global chromatin reorganization has recently been appreciated to be a hallmark of the CSC state (Gallo et al., 2015), interrogating RBBP5 in the context of epigenetic regulation is likely to reveal additional self-renewal mechanisms. Moreover, relationship between TLR4, TBK1, RBBP5, and SOX2 may explain the rapid suppression of the self-renewal signaling circuitry and tumor progression upon innate immune signaling activation. Taken together, our findings reveal that innate immune activation is a suppressor of self-renewal and may provide a unique therapeutic opportunity to compromise CSC maintenance.
Chapter 4: Discussion
4.1 Introduction

While the studies presented in this dissertation are focused on two different signaling pathways, they share a common objective. In the first set of studies, I identified the regulatory mechanism of an adhesion molecule that has been reported to be necessary for CSC maintenance and GBM progression. In this context, the restoration of the expression of miR-145 resulted in functional consequences in proliferation, self-renewal, and tumor initiation. Similarly, in the second set of studies I evaluated the response to TLR ligands and TLR expression in tumor cell populations. TLR4 expression was found to be downregulated in CSCs and its forced expression compromised the malignancy and tumorigenicity of CSCs. In both cases, decrease in the expression of core stem cell genes, SOX2, OCT4, and NANOG, is critical for the detrimental effects observed in CSCs.

4.2 Adhesion and CSC maintenance

While several adhesion molecules have been associated with CSCs and their tumorigenic potential, very little is known about the regulatory mechanisms mediating their expression. In the second chapter of this dissertation, the regulatory role of miR-145 was uncovered in CSCs. This miRNA is capable of regulating JAM-A as well as core stem cell genes, as previously reported in the literature (Xu et al., 2009). This body of work together with data previously reported from our lab (Lathia et al., 2014) makes JAM-A a key component of the cell to tumor microenvironment communication. JAM-A, now necessary and sufficient for tumor initiation, has been also targeted with blocking
antibodies with promising results in vitro. Future therapeutics should aim at translating these experiments to an in vivo setting where blocking JAM-A would render CSCs unable to establish themselves in a niche and proliferate to support tumor growth.

Junction adhesion molecules (A, B, and C) are localized mainly in tight junctions and their function has been widely explored in leukocytes, platelets, epithelial and endothelial cells (Ebnet et al., 2004). Their role, however, is not limited to establish cell to cell contacts in cells that require cell polarization; both extra and intracellular partners have been described for these molecules (Figure 4.1 and Figure 4.2). As such, JAMs can carry out additional roles like protein stabilization, scaffolding, and activating cellular processes inside cells (Ebnet et al., 2004). JAM-A was identified as preferentially expressed in CSCs as compared with non-CSCs using a surface screen a later validated to be critical for CSC maintenance (Lathia et al., 2014). Only JAM-A, but not JAM-B or JAM-C, was informative of patient survival yet the mechanism of action is not known for this protein. These observations raise a number of questions that will be addressed in the following sections.

4.2.1 What are the intracellular partners of JAM-A in CSCs?

Beyond the preferential expression of JAM-A in CSCs, the only potential role explored by our laboratory is an increased adhesion to extracellular matrix-like components.
Figure 4.1. The role of JAMs in leukocyte, platelets and endothelial cell interactions. JAMs can interact to each other or to integrins in order to associate leukocytes and platelets to endothelial cells lining blood vessels. (Taken from Ebnet, E. et al., Journal of Cell Science, 2004).
Figure 4.2. PDZ-domain-containing proteins at TJs associated with JAMs. Four TJ-associated peripheral membrane proteins, ZO-1, AF-6, PAR-3 and MUPP1, directly bind to JAM-A. ZO-1 and PAR-3 associate also with JAM-B and JAM-C. In all cases, the associations are mediated through PDZ-domain-dependent interactions. Note that the PDZ domain(s) of ZO-1 interacting with JAM-B and JAM-C have not yet been determined. (Taken from Ebnet, E. et al., Journal of Cell Science, 2004).
This was hypothesized to be due to the stabilization of integrins such as Integrin beta 1 that saw their expression increased when JAM-A was overexpressed in CSCs (data not shown). As seen in Figure 4.2, JAM-A can bind to PDZ-domain-containing proteins that are found near the periphery. The first step in order to elucidate a mechanism of action would be to investigate whether any of these interacting partners are preferentially expressed in CSCs. Interactions can be confirmed with immunoprecipitation studies as well as co immunostaining using fluorescence microscopy. Interestingly, all of the PDZ-containing proteins have additional domains that could be important for signaling purposes. For instance, ZO-1 has a SH3 domain that can serve as an anchoring point for kinases or phosphatases that need to find their substrate near the extracellular membrane. Similarly, AF-6 contains a Ras-binding domain. The Ras family of proteins is well known as small GTPases with a central role in cellular signaling. In its active form it binds GTP and activates PI3K with subsequent activation of AKT, a key signaling node in cancer and described as downstream of JAM-A in the second chapter of this dissertation. It is also important to note that ZO-1, PAR-3 and MUPP1 containing several PDZ domains that could potentially link together more than one JAM-a molecule in the membrane and stabilizing them. This can explain the increased expression of integrins that our laboratory found in the first JAM-A report (Lathia et al., 2014). In summary, several efforts should be conducted towards describing the signaling machinery downstream of JAM-A activation in CSCs and its role in glioblastoma progression. Understanding the exact pathway is important because it would help identify new signaling partners that can be targeted to render JAM-A activation inane in CSCs.
4.2.2 Is there a role for JAMs in non-CSCs?

While expression of JAM-A was consistently higher in CSCs compared with non-CSCs, the protein was still present in both populations. This was important because when blocking antibodies were used, CSCs had a dramatic effect but non-CSCs also showed a significant decrease in terms of proliferation together with an increase in cell death (Lathia et al., 2014). The magnitude of the effect might not be enormous in non-CSCs but that could be explained by the moderate rate of proliferation of non-CSCs in vitro (only 5-6 fold in a week) compared to the much higher rates in CSCs (25-30 fold in a week). This suggests then, that there could be a role for JAM-A in non-CSCs and opens up the question of whether other JAMs can also impact the proliferation and survival of this cell population. In this scenario, suppressing JAM-A function will not only affect CSCs but also non-CSCs which constitute the bulk of the tumor, making JAM-A a much desirable target. In this context, it would also be interesting to explore if JAMs would have the same role in both CSCs and non-CSCs. While stabilization of integrins was important in CSCs this might not be the case for non-CSCs. Due to the increased cellularity (a histological feature) observed in GBM and since non-CSCs compose the bulk of the tumor, we can hypothesize that JAMs can act as cell to cell bridges in this cell type. This will allow the formation of structures similar to those observed in epithelial cells where JAMs are critical for the formation of tight junctions. Understanding the different roles of JAM-A in distinct tumor populations will allow the description of mechanisms and proteins that could potentially become therapeutic targets. However, caution should be applied since JAMs can also be expressed by other cell types as
discussed in previous sections. This becomes relevant since the role of integrins and adhesion molecules has been extensively established in adult stem cell niches, such as those described in the brain.

4.3 Role of innate immunity signaling in CSC suppression

While several reports indicate that TLRs are expressed in glioma patient tissue, the cell types that express TLRs in a heterogenic tumor such as GBM and the functional role of TLRs in this setting remain open questions that need to be addressed. In which situations would it be advantageous for a cell to induce or suppress expression of a TLR? One can envision a situation where expression of TLRs in cancer cells allows the cells to respond to the stimulus by releasing pro-inflammatory cytokines that govern the behavior of endothelial cells, microglia, and infiltrating immune cells. Conversely, expression of TLRs can also be detrimental to cells expressing them in the sense that DAMPs present in the tumor microenvironment serve as ligands for these receptors. Necrosis, a hallmark of tumor progression, induces the release of a number of DAMPs that can activate downstream pathways and thus inhibit proliferation, self-renewal, and invasion. Numerous reports provide evidence for the former point of view; however, if we consider the fact that TLR3 and TLR4 stimulation can act as a negative regulator of proliferation in NPCs (Okun et al., 2009), then we can state that TLRs can be associated with non-immune roles when different signaling partners are involved. Similar questions need to be explored in order to obtain further insight into the regulation of TLRs and its association with the tumor microenvironment.
Determining the contribution of each cell population to the establishment of tumors could be achieved by labeling and lineage-tracing techniques. However, experimental challenges are anticipated based on our incipient knowledge of GBM biology. Moreover, the emergence of CSC interactions with the tumor microenvironment as a therapeutic target provides an additional layer of complexity that needs to be accounted for. By elucidating whether TLRs are involved in this communication we will contribute to the development of new therapies. Current immunotherapies target blockades in the immune system that suppress its function. If limited success is seen, a combinatorial approach with TLR modulation could benefit patients by eradicating malignant cells. Moreover, there is an essential role for TLRs in neural development and plasticity as well as an involvement in several key malignant processes in GBM. Future research should aim to move beyond the idea that TLRs only promote inflammatory pathways and the recruitment of immune cells and instead examine whether these proteins mediate a variety of cellular processes that could be pro- or anti-tumorigenic under certain spatial and temporal contexts.

4.3.1 Are all TLRs elevated in CSCs or are some reduced?

Cancer cell populations were evaluated for their capacity to respond to TLR ligands and for their expression of TLRs. Taking into account TLRs have been associated with increased inflammation and tumor progression, I anticipated cancer cells would be responsive to most TLR ligands. Yet, only TLR4 ligands were able to promote a
This unexpected finding led to the exploration of TLR family expression. While several TLRs were differentially expressed between CSCs and non-CSCs, the decreased expression of TLR4 in CSCs explained the effects seen when stimulating cells with TLR ligands. In addition, only TLR4 was informative of patient survival when patient databases were analyzed; TLR4 low levels were associated with poorer prognosis. These observations provide evidence supporting a differential expression of TLRs in CSCs. Namely, the data show TLR4 is downregulated in CSCs and this provides a beneficial lack of response to TLR4 ligands, such as damage associated ligands present in the microenvironment. Although TLR4 signaling was thoroughly explored in our studies, there are other TLRs downregulated in CSCs compared with non-CSCs. It would definitely be worth studying if these TLRs shared common signaling pathways or if each receptor is able to activate unique signaling partners.

4.3.2 In what context would TLR activation be important for tumor growth?

As previously stated, TLR activation might elicit signaling cascades that can be either pro- or anti-tumorigenic. Clearly, anti-proliferative responses will not benefit overall tumor growth overall however, the fact that different tumor cell populations (CSCs and non-CSCs) express dissimilar amounts of TLRs raises the possibility TLRs regulating proliferation and self-renewal could be activated. When conditions are appropriate for tumor expansion, i.e. nutrient and oxygen availability, TLRs promoting proliferation can be activated in both differentiated (non-CSCs) cells and stem-like CSCs. However,
when conditions are not favorable, TLRs capable of regulating non-CSC proliferation can be activated to minimize energy expenditure in cells that most likely will not be able to survive long periods of time under stress. Interestingly, it is this family of receptors that would respond to stress or damage/molecules, which are released when cells undergo necrosis (a histological hallmark of GBM). In CSCs then, the lack of expression of TLRs that regulate stem cell pathways will be critical for survival under these conditions. Conversely, TLRs that when activated actually promote the expression of core stem cell genes would be beneficial. In this dissertation I did not explore this type of interaction however based on the initial screen made on CSCs and non-CSCs several candidates appear to fall under this category (promoters of self-renewal); including TLR2 and TLR9 (higher in CSCs compared with non-CSCs) that have been recently reported in the literature to indirectly and directly be important for GBM maintenance, respectively.

4.3.3 Could targeting TLRs be an anti-tumor therapy?

In the context of TLR4, to see a potential therapeutic benefit one would have to overexpress this receptor in the CSC population of the tumor. While recent advances in delivery methods have improved their capacity to target unique tumor populations (i.e. nanoparticles and mesenchymal stem cells), this approach presents additional complications. First, forcing the expression of a protein is always more complicated than blocking its function with the use of either RNA interference or a blocking peptide. Second, and applicable to other receptors in this family, a role for TLRs in distinct
cellular populations of the brain during development and adulthood has been described. In this setting, altering or trying to modulate the expression and/or activation of different TLRs might eradicate tumor cells in detriment of physiological brain homeostasis that will not be ultimately advantageous for patients. Alternatively, we can target downstream targets that get activated upon stimulation of the receptor. In our case, the identification of RBBP5 as a regulator of stem cell gene expression was critical for CSC self-renewal and when targeted it showed dramatic effects in terms of proliferation and tumor initiation in vivo. Future experiments could analyze the expression of RBBP5 in other brain structures in order to see if it is a feasible candidate for the development of clinical approaches.

4.3.4 What role do TLR co-receptors play in determining the specificity of pro- or anti-tumorigenic role?

Although extracellular membrane TLRs bind specific ligands present in the environment to start signaling cascades inside cells by interacting with adaptor molecules, sometimes they need the assistance of co receptor molecules. There have been recent implication of these co receptors in mediating the specificity of TLRs in the response to specific ligands (Piccinini and Midwood, 2010). In Figure 4.3 one can appreciate the fact that TLR4 can recognize and be activated by a wide variety of ligands based (mainly) on the presence or absence of the CD14 and MD-2 co receptor molecules. During the course of the innate immune signaling the response to other endogenous TLR4 ligands was evaluated. For instance when I incubated non-CSCs with different size fragments
Figure 4.3. Different co-receptors mediate association with endogenous ligands. The presence or absence of specific co-receptors in the membrane of cells has been linked to the response of TLR family members to specific endogenous ligands. Not that HMGB1 is able to interact with both TLR2 and TLR4 when MD-2 is present in the membrane. (Taken from Piccinini, A.M. and Midwood, K. S., Mediators of Inflammation, 2010).
Figure 4.4. Different size fragments of hyaluronan decrease non-CSC proliferation. CD133 negative cells were incubated with different fragments and proliferation was measured at the indicated time points.
of hyaluronan (HA), I observed a decrease in proliferation with most fragments (Figure 4.4). However, CSCs also showed a decrease with HA fragments of different sizes and a consistent phenotype was never obtained. Of importance is the fact that HA fragments can stimulate TLR2 and this might have an effect in the proliferation of tumor cells. Similarly, we also incubated both cell populations with S100A8/9 signaling proteins but an effect on cell proliferation was not observed (data not shown). Moreover, when I measured the expression of CD14 in CSCs compared with non-CSCs I didn’t observe a significant difference. Although the experiments conducted did not clarify the contribution of co receptors to the effect of TLR4 stimulation in CSCs, the differential response to different agonists is very interesting. It would be worth investigating what co receptors are mediating the response to HMGB1 in the experiments presented in this dissertation as well as describing the existence of other co receptors not yet characterized.

4.4 Future therapeutics – combinatorial therapies

The body of work presented in this dissertation demonstrates that there are several dysregulated pathways in GBM, which can drive its malignancy and tumor initiating capacity. This redundancy most likely allows CSCs to activate stem cell programs even when some of the branches feeding into them are being targeted. By uncovering new stem cell pathways we are not adding to an existent already described signaling but expanding the knowledge of how cells can overcome adverse conditions. Our results show how CSCs downregulate a tumor suppressive miR-145 in order to increase the
expression of an adhesion molecule that when activated leads to AKT phosphorylation and concomitant self-renewal pathways. The second set of studies reveals CSCs have lower levels of TLR4 allowing them to proliferate despite the presence of damage signals in their microenvironment. Based on recent therapeutic failures where only one pathway was targeted, we propose a multidimensional approach that will have stem cell pathway suppression as an ultimate goal. As a paradigm, we can appreciate the efficacy of recent preclinical studies targeting multiple immune checkpoint inhibitors (Preusser et al., 2015). Thus, future studies should aim at testing combinatorial therapies that target distinct components of the tumor microenvironment that have a clear role in cell fate.
Appendix 1: List of primers used
The following primers (Integrated DNA Technologies) were used for the experiments presented in this manuscript:

**β-Actin**
Forward 5'-AGAAAATCTGGCACCACACC-3'
Reverse 5'-AGAGGCGTACAGGGATAGCA-3'

**JAM-A**
Forward 5'-ATCTGGTTTGCTATAGGCCG-3'
Reverse 5'-AGGAATGACGAGGTCTGTTT-3'

**OLIG2**
Forward 5'-AGCTCCTCAAATCGCATCC-3'
Reverse 5'-ATAGTCGTCGCAGCTTTCG-3'

**SOX2**
Forward 5'-CACACTGCCCCTCTCAC-3'
Reverse 5'-TCCATGCTGTTTCTACTCTCC-3'

**OCT4**
Forward 5'-TCTCCCATGCAATCAAAGTCC-3'
Reverse 5'-CCTTTGTGTTCCCAAATTCCTTC-3'

**NANOG**
Forward 5'-GAAATACCTCAGCCTCCAGC-3'
Reverse 5'-GCGTCACACCATTGCTATT-3'

**TLR1**
Forward 5'-CAAATGGAACAGAAGCAGG-3'
Reverse 5'-GCCTGGTACCCCCTATAGTG-3'

**TLR2**
Forward 5'-AGACCTATAGTGACTCCCAGG-3'
Reverse 5'-ACCCACACCATCCACAAAG-3'

**TLR3**
Forward 5'-TCAACTTTCTGATAAAACCTCCGCC-3'
Reverse 5'-AGATGACAGCCATTGAGACA-3'

**TLR4**
Forward 5'-TGGCAGGAGGACCAAGCAG-3'
Reverse 5'-TAAAGCCTCAGGCAGCGTTC-3'

**TLR5**
Forward 5'-TCCAGGGTTCAAGCGATTC-3'
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Ala


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