INSTRUCTIONAL CUES FOR HIERARCHY MAINTENANCE IN
GLIOBLASTOMA MULTIFORME

by

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### Instructional Cues for Hierarchy Maintenance in GBM

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Instructional Cues for Hierarchy Maintenance in Glioblastoma Multiforme

Abstract

By

KENNETH YAN

Glioblastoma Multiforme (GBM) and other cancers are challenging to treat due to their intertumoral and intratumoral heterogeneity. The hierarchical model of intratumoral heterogeneity describes the existence of a cellular hierarchy in glioblastoma with a cancer stem cell (CSC) population at the apex. These CSCs can initiate tumors and are resistant to therapies, suggesting that they are responsible for tumor recurrence. CSCs give rise to differentiated cells, which have limited tumor initiation abilities but provide a supportive CSC niche. Here, I examine the cellular cues that maintain both CSCs and the niche by tuning the relative levels of differentiation and self-renewal in GBM. I suggest Bone Morphogenetic Proteins (BMPs) as a driver of differentiation, as BMPs have been shown to promote CSC differentiation and are strongly expressed in GBM. Extrapolating from existing paradigms in development and in cancer, I then hypothesize that extracellular BMP antagonists could be the parallel drivers of CSC self-renewal.

I subsequently demonstrate that a BMP antagonist, Gremlin1, is secreted specifically by CSCs, supporting CSCs in the context of the endogenous differentiation signals provided by BMP. I promote self-renewal and increase growth of non-CSCs by overexpression of Gremlin1, and drive differentiation and
slow growth of CSCs by Gremlin1 knockdown. Finally, I examine the mechanisms downstream of Gremlin1 that drive its effects on growth and self-renewal, as well as the mechanisms upstream of Gremlin1 that promote its differential secretion by CSCs. Downstream of Gremlin1, cell proliferation effects are mediated by p21 inhibition, and self-renewal is partly mediated by activation of Wnt signaling. Upstream of Gremlin1, I identify XBP1, a pro-survival factor in the unfolded protein response, as a CSC-specific signal that might be promoting Gremlin1 expression. In the course of this thesis, I identify a novel molecular target, Gremlin1, as well as several associated signaling pathways that could be targeted to enhance current differentiation-based therapies and benefit GBM patients.
Chapter 1

Introduction

In my training in Dr. Jeremy Rich’s laboratory, I have had the opportunity to contribute to a number of different reviews relating to GBM cancer stem cells (CSCs). This chapter is adapted from the following two reviews, one of which has been published in Current Opinion in Neurology, and the other of which is to be submitted to the Journal of Neuro-oncology:


*Authors contributed equally to this work.
1.1. The Heterogeneity of Cancer

Cancers are difficult to treat due to their inherent heterogeneity. This heterogeneity is both intertumoral (between patients) as well as intratumoral (within a single patient). Intertumoral heterogeneity might explain how one person might be more or less responsive to a given therapy. Meanwhile, intratumoral heterogeneity at the level of a single patient could explain how a subpopulation of cells in a tumor could resist therapy and be responsible for cancer recurrence in the patient. In addition, an element of intratumoral heterogeneity is a tumor’s ability to use cells in the normal microenvironment to benefit its own growth.

The classical example of intertumoral heterogeneity impacting treatment is in acute myelocytic leukemia (AML). AML can be classified into a number of different subtypes, one of which is acute promyelocytic leukemia (APL), characterized by a t(15;17) translocation [1]. The t(15;17) translocation is between the PML gene on chromosome 15 and the retinoic acid receptor (RARα) gene on chromosome 17. This translocation results in an abnormal gene product that suppresses an anti-proliferative gene in a dominant-negative fashion, resulting in the development of leukemia [1]. However, in the presence of high levels of retinoic acid, the suppression is released, causing leukemic cells to differentiate [2]. Therefore, in APL, patients may be treated by the administration of retinoic acid [3].

Intratumoral heterogeneity is also present in AML. One level of intratumoral heterogeneity is provided by the supporting normal cells in the tumor
microenvironment. In AML, bone marrow stromal cells secrete TGFβ that promote tumor growth [4], and AML cells can integrate into the endothelium, which can serve as a tumor reservoir [5]. Another level of heterogeneity is provided by the existence of a CSC population, leukemia-initiating cells [6]. These cells represent a subpopulation of cells in AML that are able to self-renew and differentiate into all forms of AML, and propagate tumors in non-obese diabetic / severe combined immunodeficient (NOD/SCID) mice.

1.2. Intertumoral Heterogeneity of Glioblastoma Multiforme

The observations of tumor heterogeneity in AML may be extended to solid tumors. Among solid tumors, glioblastoma multiforme (GBM) is a prime example of a cancer that exhibits both intertumoral and intratumoral heterogeneity. As such, this thesis will use GBM as the primary model to study cancer heterogeneity. GBM, or World Health Organization grade IV astrocytoma, is a uniformly fatal primary brain tumor typically presenting in patients over the age of 40. For nearly half a century, the standard-of-care for GBM was maximal surgical resection followed by radiotherapy [7]. Temozolomide, a nitrosourea-based chemotherapeutic agent, was added in the 1990s with modest survival benefits [8]. Despite incremental discoveries since then, treatment for GBM has remained largely unchanged. Furthermore, tumor recurrence is the rule and patient prognosis has continued to be dismal with a median survival of about 15 months with very few long-term survivors [7].
In the early 2000s, it was discovered that response to temozolomide chemotherapy is related to the promoter methylation status of MGMT (O\textsuperscript{6}-methylguanine DNA methyltransferase), a key DNA repair gene [9], suggesting that there is intertumoral heterogeneity among GBM patients which can inform therapies. As such, in the past few years, there has been a strong push to characterize GBM on the molecular level to obtain a better understanding of this molecular heterogeneity among GBM patients. The Cancer Genome Atlas (TCGA) project represents a large-scale analysis of over 500 patients that identified three critical signaling pathways involved in different subsets of GBM patients – the p53, receptor tyrosine kinase (RTK) and Rb gene networks [10]. At the same time, a sequencing analysis in a distinct patient set identified recurrent mutations in isocitrate dehydrogenase 1 (IDH1) as genetic lesions in certain secondary GBMs that correlated with better patient prognosis [11]. Through TCGA data, GBM has been classified into three major groups – proneural, mesenchymal and classical subtypes – based on genetic alterations in PDGFRA/IDH1, NF1, and EGFR [12]. A subset of patients in the proneural subgroup were found to have a CpG island methylated phenotype (G-CIMP) that was linked to the IDH1 mutations and better prognosis [13]. Finally, most recently, the TCGA dataset was used to reveal further novel mutations and key somatic gene rearrangements in GBM [14].
1.3. Intratumoral Heterogeneity of GBM

These molecular characterizations and classifications represent major breakthroughs in GBM biology, but have not translated into personalized therapies or drugs in clinical trials. A major reason for this is the presence of additional intratumoral heterogeneity at the level of a single patient. In the context of tumor subgroups identified by TCGA, it has even been suggested that a single tumor might contain genetically distinct regions of proneural and mesenchymal subtypes based on extrinsic factors such as macrophage infiltration [15].

As in AML, another level of heterogeneity is also provided by the adaptation of cancer cells to the normal microenvironment provided by the host organ – in the case of GBM, the brain. A normal brain contains a number of different cell types – that is, astrocytes, neurons, oligodendrocytes, neural stem cells, and microglia – as well as cells found in and around blood vessels, including endothelial cells, pericytes, lymphocytes, macrophages, red blood cells and platelets. Collectively, these cells comprise a tumor "niche" for GBM. GBM hijacks the normal cells in the tumor niche for their own benefit. For example, macrophages [16], endothelial cells [17] and reactive astrocytes [18, 19] have all been shown to secrete factors to promote GBM growth. It has even been shown that tumor cells themselves can give rise to abnormal niche elements, such as pericytes [20] and endothelial cells [21, 22].

Lastly, in addition to the heterogeneity provided by the host organ, the existence of GBM CSCs provides an additional level of heterogeneity. Similar to
the observations in AML, CSCs within GBM are defined functionally by their ability to self-renew and propagate tumors [23]. In addition, GBM CSCs are resistant to both treatment modalities of radiation [24] and chemotherapy [25]. Together, these two observations suggest that CSCs might represent the tumor population responsible for GBM recurrence following therapy. In the next few sections, I will provide a background of the history, properties, and isolation of GBM CSCs.

1.4. History of Cancer Stem Cells

Two general models can explain the heterogeneity of cancerous cells within a given tumor such as GBM [23]. In the stochastic model, heterogeneity is derived by random mutation, and tumors are comprised of a diverse pool where every cell is capable of tumor initiation but at a random, low frequency. In the hierarchical model, the tumor cells exist in a hierarchy with CSCs at the apex. The model suggests that with their ability to self-renew as well as to differentiate into restricted lineages, CSCs are the only cells capable of tumor initiation within a given tumor. While CSCs have a number of different properties that I will discuss in detail below, this capability of tumor initiation is the sole defining property of a CSC.

Prior to the isolation of putative CSCs in solid tumors, similarities between cancer cells and normal stem cells had been apparent, such as the potential for self-renewal and the expression of canonical stem cell signaling pathways such as Wnt, Shh and Notch [23]. As mentioned, CSCs were initially explored in the
context of hematological malignancies such as AML [6], and these findings were later extended to solid tumor models. In the early 2000s, CSCs were first isolated in solid tumors, initially in breast, but followed closely by GBM [26-28]. Following these initial observations, CSC populations were then isolated in a number of different solid tumors, including colon cancer, ovarian cancer, melanoma, osteosarcoma, and pancreatic cancer [29-33].

1.5. Properties and Pathways Governing Cancer Stem Cells

As mentioned earlier, the defining property of CSCs is their ability to initiate and recapitulate a tumor in vivo (Fig. 1.1). In practice, this property is assessed by injecting prospective matched CSCs and non-CSCs into an in vivo model, and determining if and when a tumor forms in the model organism. In addition, in order to initiate and recapitulate a tumor, CSCs need to possess the ability to self-renew, to generate more CSCs, and to differentiate into the cells that comprise the majority of the tumor bulk. Self-renewal is often assessed using sphere formation assays, where CSCs are plated in single cell-growth conditions and allowed to self-renew to form tumorspheres (or in the case of GBM CSCs, neurospheres). Differentiation state is frequently assessed by evaluating stem cell marker expression.

There are also many other properties that have been found to be unique to CSCs, though none can be considered strictly defining of CSCs. Importantly, some of these properties promote tumor growth and therapeutic resistance, increasing the attractiveness of CSCs as therapeutic targets. In GBM, CSCs
contribute to tumor angiogenesis by secretion of pro-angiogenic growth factors [34] and possible lineage plasticity towards vascular lineages [20-22]. In addition, while conflicting reports exist [35], CSCs are thought to play roles in GBM invasion [36, 37]. This is further supported by the fact that pluripotency factors Nestin and Oct4 are associated with invasion in GBM [38, 39]. Furthermore, CSCs are resistant to current treatments for GBM, a property that, when combined with their inherent ability to initiate tumors, suggest CSCs as drivers for tumor recurrence [24]. Chen and colleagues modeled this phenomenon in a mouse model of glioma with a Nestin-ΔTK-GFP transgene. The GFP+ cells were suggested to represent a CSC population that remained following temozolomide chemotherapy with additional therapeutic benefit of targeting tumors with the combination of temozolomide and gancyclovir to target the putative CSCs [25]. In addition, a mathematical model of GBM radiotherapy and recurrence predicted that surviving CSCs increased their rate of self-renewal to maintain a tumor following radiotherapy [40]. These and other studies further demonstrate that CSCs may contribute to our current failure to achieve cures in patients, supporting the development of CSC-specific targeting strategies.

Initial models of GBM CSC molecular regulation have been based on neural stem cell biology, the probable normal cellular correlate. Indeed, CSCs share many properties with normal stem cells or neural stem cells. For instance, CSCs express many stem-cell related transcription factors, including three of the Yamanaka factors [41] Oct4, Sox2 and c-Myc [42], in addition to Olig2 and Nanog. In addition, CSCs are associated with tumor niches similar to stem cell
niches (described in Section 1.7). Finally, CSCs are governed by pathways active in stem cell and brain development, including Notch [43], Wnt [44], Hedgehog [45, 46], bone morphogenetic protein (BMP) [47], transforming growth factor-β (TGFβ) [48, 49], and receptor tyrosine kinase (RTK) pathways [50-52] (Fig. 1.2), which I elaborate below. Targeting components of these CSC-specific pathways might provide new avenues for GBM treatment.

Receptor Tyrosine Kinases

Receptor tyrosine kinases have long been implicated in GBM biology. Epidermal Growth Factor Receptor (EGFR) in particular is widely studied because it is mutated or amplified in nearly half of GBM patients [10]. Alternatively, patients express the constitutively active EGFRvIII mutant [53]. EGFR and PDGFRA mutations are defining genetic aberrations for two GBM TCGA subtypes [12]. EGFR has been implicated in GBM CSC biology in some reports [54, 55] – however, there is increasing evidence that EGFR amplification is a tumor-wide phenomenon and CSCs instead preferentially activate other RTK-mediated pathways. For instance, PDGFRβ is thought to modulate CSCs through STAT3 activation [50], and Eph receptors EphA2 [51] and EphA3 [52] have both been implicated in CSC biology.

One particular receptor that has generated much interest in GBM CSCs is MET, or hepatocyte growth factor receptor. Following EGFR inhibition, cells with high c-MET expression arise that express CSC markers and stem-like phenotypes [56]. In CSCs, MET has been shown to activate downstream Akt
signaling and induce the pluripotency factor Nanog [57, 58]. It has also been suggested as a marker for proneural and mesenchymal CSCs [59].

**TGFβ and Bone Morphogenetic Proteins**

TGFβ and BMPs are both part of a superfamily of extracellular ligands that share a common cysteine knot motif and signal through SMAD protein phosphorylation. However, despite their similarities, TGFβ ligands have been widely shown to promote CSC maintenance while BMPs tend to induce differentiation. Specifically, TGFβ promotes CSCs through a number of different pathways, including ID proteins [48], LIF [49], and Sox4-mediated Sox2 induction [60]. BMPs, despite being expressed by GBM cells and infiltrating neural stem cells [61], induce differentiation of CSCs into an astrocyte-like lineage [47]. The effects of BMPs are partly mediated through the transcription factor Atf3 [62]. BMP signaling in CSCs will be further described in Chapter 2.

**Wnt**

The Wnt pathway has been heavily studied as a stem cell maintenance pathway and plays a major role in other cancers such as familial colon cancer. A number of studies have begun to implicate Wnt signaling in GBM CSC maintenance as well. Key components of canonical Wnt signaling such as Wnt receptors Frizzled4 [44] and LGR5 [63], as well as signaling mediator Dishevelled2 [64], have been directly linked to CSC biology.
Additionally, a number of other mediators have been shown to effect CSC phenotypes by impinging on Wnt signaling. For instance, the MET receptor can activate Wnt signaling [65], and FoxM1 can function to promote β-catenin nuclear localization [66]. In addition, in a recent large-scale epigenomic analysis of CSCs, Rheinbay et al. identified a number of differentially regulated transcription factors in CSCs compared to astrocytes and neural stem cells, including ASCL1 [67]. By repressing the negative Wnt regulator DKK1, ASCL1 activates Wnt signaling to promote CSC maintenance.

**Hedgehog**

Hedgehog downstream signaling is mediated by the GLI family of transcription factors, which are so-named because of their discovery in gliomas. Historically, Hedgehog signaling has been more strongly implicated in medulloblastoma, a childhood brain tumor, rather than GBM. Medulloblastoma is a component of basal cell nevus syndrome, which is caused by mutations in the Hedgehog receptor, Patched [68]. However, in GBM, Hedgehog inhibition by Cyclopaamine has been shown to deplete CSCs in isolation [45] as well as in conjunction with EGFR inhibition [46]. The stem cell maintenance factor Nanog is thought to be upstream of GLI in CSCs [69] and Hedgehog signaling itself plays a role in CSC proliferation [70].
Notch signaling is mediated by cell-cell interactions, in that Delta and Jagged ligands on one cell interact with Notch receptors on a neighboring cell. This pathway is an attractive target in CSCs because it is readily targetable with γ-secretase inhibitors, which are used for treatment in Alzheimer’s disease. Using such inhibitors, Notch has been implicated in GBM CSC maintenance [71, 72] as well as the radioresistance phenotype of CSCs [43]. Another recent study suggested that high Notch signaling and sensitivity to γ-secretase inhibitors might be specific to CSCs in the proneural subtype [73]. In addition, two key CSC signaling nodes, STAT3 and NFκB, have been shown to be upstream of Notch1 in CSCs [74].

1.6. Isolation of Cancer Stem Cells

There are two opposing methods for isolation or derivation of CSCs from primary GBM specimens (Fig. 1.3). In one school of thought, GBM CSCs are derived via in vitro culture of GBM cells in serum-free media with the addition of growth factors, such as fibroblast growth factor-2 (FGF2) and epidermal growth factor (EGF), that are permissive for growth of neural stem cells in culture and allow for the maintenance of multipotent, self-renewing, and proliferative characteristics [75, 76]. In this method, the growth of tumor spheres are induced epigenetically in response to growth conditions in culture; these cells also co-express neuronal and glial markers and display the plasticity observed in normal neural stem cells in comparable regions of the central nervous system. It has
been shown that these culture conditions do indeed promote the stem cell qualities of CSCs [77]. However, a number of labs, including the Rich Lab, are opposed to this method as it involves artificially modifying cells in tissue culture. In addition, this method does not preserve a non-CSC population.

Another method of CSC isolation involves the sorting of primary tumors for CSC marker expression. This method has the advantage of the acute isolation of CSCs from an in vivo model; however, there is extensive debate surrounding marker choice and there is likely no perfect marker for CSCs. When Bonnet and Dick first isolated AML-initiating cells in the 1990s, they isolated cells by positive expression of the cell surface marker CD34 but not CD38 [6]. Engraftment of CD34⁺CD38⁻ cells in NOD-SCID mice led to the homing of the stem-like tumor cells to the bone marrow and widespread disease reflective of human AML [6]; the self-renewal capacity of these tumor initiating cells were confirmed through serial in vivo transplantations [78]. In addition, initial studies in breast also identified CSCs through the expression of cell surface markers, CD44 and CD24 [26]. The study showed that CD44⁺/CD24⁻ cells derived from primary patient tumors successfully engrafted in the NOD-SCID model and recapitulated the phenotypic heterogeneity observed in the initial primary tumors.

The first marker that was proposed to isolate CSCs in GBM was CD133, or Prominin-1, and this marker is still in use today. CD133 is a cholesterol-binding cell surface protein that has been used as a marker for hematopoietic stem cells [79]. The utility of CD133 has been controversial and dependent on context, but a functional role for CD133 has recently been elucidated in GBM
CSCs, as a regulator of the PI3K-Akt pathway via its interactions with the p85 subunit of PI3K [80]. In addition, Herold-Mende and colleagues examined the clinical relevance of CD133 by interrogating the expression levels of CD133 in a cohort of 95 glioma samples of different WHO grades. CD133 expression by immunohistochemistry correlated significantly with increasing malignancy and multivariate survival analysis strongly suggested an inverse correlation with overall survival time, independent of WHO grade, age, and extent of surgical resection [81]. Lastly, as a cell surface protein, CD133 has been targeted with antibodies in preclinical studies and a vaccine against CD133 (ICT-121) is entering clinical trials.

The CSC state is a plastic phenotype and, as mentioned, there is no single cellular marker that is universally informative for CSCs. As such, the study of CSCs requires functional validation and use of patient derived tumors with no or limited time in culture to prevent clonal drift. Previous members in the Rich Lab [24, 82, 83] and I have performed the functional validation of CD133 as a CSC marker in several of our tumor specimens for use in our studies. In my hands, CD133-positive populations represented less than 5% of the cells in our specimens. CD133-positive populations expressed stem cell transcription factors Sox2, Olig2 and Nanog, had an increased ability for self-renewal, and initiated tumors in immunocompromised mice (Fig. 1.4).

While CD133 will be used to prospectively isolate GBM CSCs in the remainder of this thesis, it is worth mentioning a few other proposed CSC markers here. The initial isolation of CSCs also described Nestin, an
intermediate filament protein that is present in dividing cells during early stages of CNS development, as a potential CSC marker [27]. CD15, or stage-specific antigen 1 (SSEA-1) is a neural/stem progenitor marker that plays a role in the adhesion and migration of pluripotent stem cells, and has been proposed as an additional marker of GBM CSCs [84]. Another group identified a perivascular-associated CSC population that is promoted by TGFβ signaling, which expresses CD44 and ID1 [48]. In addition, other studies have suggested the use of A2B5 [85], integrin-α6 [86] and L1CAM [87] as potential CSC markers. While all of these markers have demonstrated their own utility, there is no end-all marker for CSC enrichment.

1.7. The Cancer Stem Cell Niche

No stem cell exists in isolation. Similar to normal stem cells, GBM CSCs exist within the tumor niche, which includes not only microenvironmental stressors, but also a wide variety of cell types. Recently, there has been increasing interest in studying the components of the CSC microenvironment to uncover novel targets. The characteristics of the tumor niche that influence CSCs are considered below.

Microenvironmental factors influencing CSC biology

CSCs are influenced by microenvironmental factors that are present within their niche, including acidic stress, nutrient deprivation and hypoxia [88-90]. Of these, hypoxia, which is a result of the disordered vasculature within GBM, is the
best characterized. While physiological oxygen levels in the body are between 1-7%, areas within GBM might fluctuate to significantly lower levels. The hypoxia causes regions of pseudopallisading necrosis [91]. These areas of necrosis are increasingly thought to represent a niche for CSCs, as hypoxia has been shown in other systems to prevent the differentiation of normal stem cell populations [92]. In addition, hypoxia has been linked to MGMT expression, suggesting that cells in hypoxic regions of GBM might be better suited to evade treatment [93].

Consequently, CSCs have been shown to cluster in hypoxic regions [94], and hypoxia has been shown to increase the expression of stem cell markers [95] and promote a more stem-like phenotype [90]. Therefore, one potential area of therapy against CSCs could be to target the hypoxic niche. The hypoxic response is canonically regulated by hypoxia inducible factors (HIFs). Of the three isoforms of HIF-alpha subunits, HIF1α and HIF2α have both been implicated in CSC biology, suggesting that they might play overlapping roles in GBM. HIF1α has been shown to promote CSC expansion [96] and to prevent BMP-mediated differentiation in CSCs [97]. This effect might be partially mediated through Notch signaling [98]. Meanwhile, following hypoxia, HIF2α is preferentially induced in CSCs compared to the differentiated fraction, and promotes CSC self-renewal [83]. The preferential induction of HIF2α in CSCs is due in part to epigenetic regulation by MLL1 [99].
Supporting cells in the CSC niche

As mentioned earlier, the cancer niche is a heterogeneous mixture of cells of many types, including endothelial cells, pericytes, neurons, reactive astrocytes, and tumor-associated macrophages. When considering CSCs, though, there is the added complication that certain components of the CSC niche are comprised of the normal cells from the host organ, whereas other elements of the niche are tumor cells derived from the CSCs themselves. Indeed, even certain “normal” cell types such as pericytes and endothelial cells might be CSC-derived [64, 65, 66], as they have been shown to possess the same genetic abnormalities as tumor cells suggesting a neoplastic origin.

A number of supporting cell types found in the GBM CSC niche play roles in CSC maintenance, often through the elaboration of secreted factors. First, cells associated with the vasculature are the most studied cells that affect CSC biology. CSCs have been shown to promote angiogenesis [34], and in turn, blood vessels are thought to create a “perivascular niche” for CSCs by expressing IL-8 and Notch ligands [17, 100]. GBM CSCs even express receptors for vascular endothelial growth factor (VEGF). Secondly, tumor-associated macrophages/microglia (TAMMs) have been shown to interact with GBM CSCs. CSCs specifically recruit these cells and induce an immunosuppressive phenotype [101, 102]. Correspondingly, TAMMs promote CSC growth through secretion of TGFβ ligands [16]. Lastly, differentiated tumor cell populations appear to maintain CSCs, by producing ligands such as interleukin-6 (IL-6) [103] and extracellular matrix components such as laminins [104] that promote CSC
proliferation. These initial observations suggest that when targeting CSCs, it is important to consider the surrounding environment. However, while we are making progress in characterizing the microenvironmental influences, cell types and secreted factors that comprise the CSC niche, there is still a lot that remains to be explained.

1.8. Conclusion

Both intertumoral and intratumoral heterogeneity hinder our ability to treat cancer patients. In GBM and many other solid tumors, one major component of intratumoral heterogeneity is the existence of CSCs, at the peak of a hierarchy of cancer cells [23]. CSCs in GBM have tumorigenic properties such as angiogenic and invasive ability [34]. In addition, they are resistant to therapy [24, 25] and able to initiate tumors. These dual properties suggest a role for CSCs as the cells responsible for GBM recurrence, and, consequently, patient death. Therefore, there is a need to target GBM CSCs to ultimately treat this deadly disease.

In finding treatments against CSCs, many studies initially focused on the pathways that are intrinsic to CSCs, and often present in normal stem cell correlates. More recently, researchers have started looking into the microenvironment in which the CSCs call their home. This microenvironment is composed of stressors such as hypoxia, a number of different cell types, and likely many secreted factors, both paracrine and autocrine, that have yet to be uncovered. In the next chapters, I examine the how the interplay between some of these secreted factors is involved in maintaining the hierarchical structure of
GBM. In addition, I determine how one particular secreted factor, Gremlin1, affects CSC biology by impinging on cell-intrinsic pathways.
Figure 1.1. Characteristics of CSCs. Defining and other functional characteristics of CSCs.
Figure 1.2. Molecular pathways in GBM CSCs. A schematic of some of the molecular pathways regulating CSC biology.
Figure 1.3. Derivation of GBM CSCs. In one school of thought (above), CSCs are derived from prolonged tissue culture in growth factor media. While straightforward, this method involves artificial modification of cells in tissue culture, representing a potential departure from the biology in the original tumor. The other method of CSC isolation (below) involves acute sorting for a CSC marker. This method is more difficult, and there is no end-all marker for identification of CSCs, but the CSCs isolated are more similar to the original tumor.
Figure 1.4. Validation of CD133 as a marker for glioma CSC enrichment. (A) CD133+ fractions following sorting by CD133 magnetic beads. (B) Western Blot demonstrating differential expression of the stem marker Olig2 following sorting for CD133. RT-PCR for (C) Sox2, (D) Olig2 and (E) Nanog demonstrating differential expression in CD133+ and CD133- populations. (F-H) In vitro limiting dilution assays demonstrating increased sphere formation potential in CD133+ populations. (I-K) Quantification of F-H. (L,M) In vivo limiting dilution assay demonstrating decreased tumor latency and increased tumor formation in immunocompromised mice for CD133+ populations. *, p<0.05; **, p<0.01; ***, p<0.001.
Chapter 2
BMP Signaling in the GBM Tumor Hierarchy

2.1. Glioma Cancer Stem Cells at the Apex of a Hierarchy

The hierarchical model of intratumoral heterogeneity described in the previous chapter posits that the tumorigenic population within GBM is arranged in a hierarchy with CSCs at the peak. Within the hierarchy, CSCs are capable of tumor initiation and are responsible for tumor phenotypes such as angiogenesis, invasion and therapeutic resistance. Conversely, differentiated cell populations are unable to readily initiate tumors and have diminished abilities to drive these same tumor phenotypes.

Reports of CSC isolation describe CSCs as rare cell populations within tumors, with as few as 1 in 54000 cells with tumor-initiating characteristics [26-29, 32]. In the context of the hierarchical model, this observation suggests that CSCs rapidly recreate cellular hierarchies in vivo. However, this is challenging to reconcile with cell autonomous evolutionary models of cancer growth, which suggest that clones with selective growth advantages will dominate the tumor bulk. Two questions come to mind, then; why do CSCs promote the continuous creation of a hierarchy, and how does this process occur?

Recently, studies have provided insight into the first of these questions – perhaps the continuous creation of a hierarchy is driven by the ability of non-CSC differentiated cells to serve as essential components of the CSC niche. A relationship such as this is present in Drosophila, where multipotent somatic cells
provide a stem cell niche in the normal testes [105]. It is also present in the normal human colon, where LGR5+ stem cells give rise to Paneth cells that provide ligands for stem cell proliferation [106]. In GBM, differentiated cancer cells have been shown to provide IL-6 ligands and extracellular matrix components (laminins), both of which then serve to promote CSC maintenance [103, 104]. Lastly, GBM CSCs have been demonstrated to have the ability to differentiate into endothelial cells or pericytes, components of the well-characterized vascular niche for CSCs [20-22].

From these preliminary studies, it is evident that there is an incentive for continuous creation of a hierarchy with GBM CSCs at the apex and supportive differentiated cells below. However, the second question posed above remains to be answered – how does a given tumor maintain a balance between CSCs and their corresponding supportive niche elements? In the coming chapters, I examine the paracrine and autocrine effectors within the CSC niche that are responsible for maintaining this essential balance. Targeting these effectors and disrupting this balance could represent a new modality by which to treat GBM.

2.2. Bone Morphogenetic Protein Signaling: An Introduction

In order to maintain both CSCs and their differentiated progeny, I hypothesized that there must be a driver of CSC differentiation and a parallel driver of self-renewal present within GBM. By controlling the relative levels of these parallel drivers, a GBM can thereby control the relative levels of CSCs and supportive differentiated cells. BMPs, particularly BMP2, BMP4, and BMP7, are
well-characterized in GBM as drivers of CSC differentiation into an astrocyte-like lineage, causing a corresponding decrease in proliferation and tumorigenicity [47, 61, 62, 107]. However, these same BMPs are paradoxically expressed within GBM [108, 109], suggesting that they might play a functional role as a driver of CSC differentiation within the CSC hierarchy.

BMPs represent a group of roughly 20 structurally-related cytokines within the TGF-β superfamily of cysteine knot proteins [110-112], and they were first identified as peptides that induced bone formation. BMPs are expressed as large precursor proteins with an N-terminal signal peptide and a prodomain that is cleaved following secretion. Active BMPs are homo- and heterodimers linked by disulfide bonds and bind to complexes composed of type I and type II BMP receptors. Canonical BMP signaling involves the binding of BMPs to preformed heteromeric receptor complexes. Upon receptor binding, the type II receptor activates the type I receptor, which then phosphorylates receptor regulated SMAD proteins (R-SMADs) – SMAD1, SMAD5 and SMAD8 (Fig. 2.1). Phosphorylation of R-SMADs causes a conformational change that leads to their interaction with SMAD4 (Co-SMAD). The activated SMAD complex then translocates into the nucleus to activate transcriptional targets, such as Runx2, Xvent2, and ID proteins. Non-canonical BMP signaling is initiated when the BMP ligand binds to a homomeric type I or type II receptor, which then causes secondary formation of a heteromeric complex. This complex then activates MAPK, ERK1/2, c-Jun or p38 kinases, which can activate downstream effectors independently or alongside SMAD phosphorylation [113].
BMP signaling is modulated tightly in a tissue- and developmental stage-specific manner by several different mechanisms, one of which is receptor expression and affinity [111, 112, 114]. For instance, BMP signaling through BMPR1b will induce the differentiation of mesenchymal stem cells (MSCs) into osteoblasts [115], or the differentiation of GBM CSCs into astrocytes [47], while BMP signaling through BMPR1a induces adipogenic differentiation in MSCs, and proliferation in CSCs [107]. In addition, BMPs are regulated by a number of intracellular, extracellular, and cell-surface regulators. Cell-surface regulators of BMP signaling include co-receptors that potentiate signaling such as Endoglin and Betaglycan [116, 117] and decoy receptor BAMBI which inhibits signaling [118]. Intracellular regulators most notably include inhibitory SMAD proteins, SMAD6 and SMAD7 (I-SMADs), which bind to type I BMP receptors and block phosphorylation of R-SMADs [110-112].

This thesis focuses on extracellular BMP antagonists, which directly bind to BMPs and obscure the epitopes of the BMPs that interact with their corresponding receptors [119, 120]. Similar to the BMP ligand, BMP antagonists exist as dimers and contain cysteine knot domains [112, 114]. The antagonists are classified into several groups based on the number of cysteines in this domain. The Dan/Cerberus (CAN) family, which contains eight cysteines (the same number as the BMP ligands themselves), includes Dan, Cerberus, Gremlin1, Gremlin2, and others [121]. A full list of known BMP extracellular antagonists can be found in Table 2.1.
2.3. BMP Signaling in Normal Neural Development

Before examining BMP signaling in GBM CSCs, it is important to consider the intricacies of BMP signaling in neural stem cells (NSCs), the closest normal correlate to GBM CSCs, and nervous system development. In general, BMP and antagonist feedback loops are apparent throughout development, where both BMPs and extracellular antagonists are secreted to fine-tune developmental events. The classical example of this is during gastrulation in the *Xenopus* embryo, where the BMP antagonists Cerberus, Chordin, Noggin, and Follistatin are secreted by the dorsally-located Spemann organizer to create a BMP gradient that will dictate embryonic patterning in the formation of the three germ layers – endoderm, ectoderm, and mesoderm [122]. In addition, a BMP and antagonist feedback loop serves to trigger termination of limb development [123], and spatially restricted Gremlin1 secretion blocks BMP signaling in a specific zone of the Wolffian duct to promote ureteric bud formation during kidney development [124].

BMPs play roles during all stages of neural development. In neurulation, a BMP gradient specifies cell fates within the neural tube [125]. Furthermore, active BMP signaling is required for neural crest formation and maintenance, while BMP inhibition is required for preplacodal ectoderm formation [126, 127]. Crossveinless-2 (Cv2), though typically a BMP antagonist, acts as an enhancer of BMP signaling for neural crest induction. BAMBI, the BMP decoy receptor, acts as an inhibitor of BMP signaling in preplacodal ectoderm formation [126].
After neurulation, the nervous system begins as an epithelium composed of rapidly proliferating precursor cells (radial glia) that maintain connections to both the ventricles and the pial surface [128]. These prenatal NSCs eventually give rise to neurons, astrocytes, and oligodendrocytes in the adult, in addition to adult NSCs in the subventricular zone (SVZ) [76, 129, 130]. At earlier stages in neural development, BMP specifies neuronal fates of radial glia in the presence of neurogenin-1 (Ngn1) [131]. Later in development and continuing into birth, BMP specifies astroglial fates in the presence of high Jak-STAT signaling [132]. In adult mouse models, neurogenic regions are limited to the SVZ and the subgranular zone (SGZ). In the SVZ, ependymal cells secrete Noggin to direct the generation of neurons from SVZ precursors [133]. Similarly, in the SGZ, BMPs promote quiescence and help maintain the neural stem cell population, while Noggin promotes neuronal expansion [134]. All-in-all, from these observations, it is very clear that BMPs play a highly location-specific and stage-specific role in neural development.

2.4. BMP Signaling in Cancer

The role of BMPs in cancer is heavily debated, with contradictory reports as to whether BMPs promote or inhibit tumor formation and maintenance [135]. This is not surprising, given the tremendous diversity in BMPs roles in development. Generally, BMPs 2, 4, and 7 have been demonstrated to impact four cancer phenotypes – proliferation, invasion, angiogenesis, and differentiation. An overview of BMP effects in cancer cells is provided in Table
2.2. BMPs tend to inhibit proliferation in tumor cells [136, 137], often through the activation of p21 [137-139]. However, there are reports of BMPs promoting proliferation [107, 140]. Analogous conflicting data exist for BMP-mediated invasion and migration [141-146], as well as angiogenesis [147]. In colon cancer, osteosarcoma, and GBM, BMPs promote differentiation, and in turn, decrease tumor growth [47, 148, 149].

BMP antagonists have been largely neglected as players in tumor biology, and are often used as experimental tools to study the roles of BMPs [143, 150]. However, BMP antagonists themselves are starting to be implicated in cancer, with conflicting roles in tumorigenesis. For instance, *Grem1* mRNA is overexpressed in lung, ovary, breast, colon, kidney, and pancreatic cancers in addition to mesothelioma, suggesting a role in oncogenesis [151, 152]. Supporting this notion, Gremlin1 has been demonstrated to play an essential role of the tumor niche in many cancers. It has been shown to be secreted by stromal cells and tumor-associated vasculature, and causes a proliferative phenotype in skin cancer [153, 154]. In lung cancers, Gremlin1 is overexpressed and promotes cell proliferation, migration, and invasion [155, 156]. The BMP antagonist Noggin is strongly implicated in skin tumorigenesis [157, 158]. In addition, Noggin involved in a feedback loop in prostate cancer, promoting proliferation and osteolytic bone metastases [159, 160]. The antagonist Coco provides a niche to allow for lung metastases of breast cancer cells [161]. Lastly, the antagonist Follistatin is implicated in the initial stages of gastric transformation [162].
In contrast, other works suggest that these antagonists have tumor-suppressive properties. For example, studies have demonstrated inactivation of Gremlin1 in lung, breast, prostate, and bladder cancer, and Gremlin1-mediated transcriptional activation of p21 [163, 164]. Furthermore, Chordin is downregulated in ovarian tumors and inhibits BMP4-mediated invasion when overexpressed [165]. Similarly Sclerostin-Domain Containing-1 (SOSTDC1) is downregulated in renal clear cell adenocarcinoma and inhibits proliferation when reintroduced [166]. These conflicting studies indicate that there is still much to be determined about the role of BMP and its antagonism in cancer. Furthermore, the role of BMP antagonists in GBM is completely unknown.

2.5. BMP Signaling in the GBM Cancer Stem Cell Hierarchy

In this chapter, I establish the need for continuous generation of a cellular hierarchy in GBM, with differentiated cells providing niche signals to the tumorigenic CSC population at the apex. One outstanding question is the mechanisms that promote the formation and maintenance of this hierarchy. In order to maintain both stem and differentiated populations, I suggest that there might be a driver of differentiation and a parallel driver of stem cell maintenance.

BMPs are the ideal candidates for the drivers of differentiation, as they promote differentiation in normal neural stem cells in addition to GBM CSCs. Furthermore, they are paradoxically expressed or even elevated in GBM despite these supposed negative effects. In order to complete this proposed model, there needs to be a parallel driver of stem cell maintenance that will maintain the CSC
population in a BMP-rich environment. Based on what we know from development and from cancer, extracellular BMP antagonists are likely candidates for this driver. Similar BMP-antagonist relationships are present in development to regulate patterning in gastrulation and neurulation, and to fine-tune signals in limb and kidney development [123, 124]. These relationships are also present in cancer in melanoma and prostate cancer [157, 159]. Therefore, I hypothesize that a BMP antagonist is secreted to maintain GBM CSCs in the context of pro-differentiation signals from BMPs. In the next chapter, I explore this hypothesis in detail.
Figure 2.1. Canonical BMP signaling and its regulation. BMPs signal by binding to heterodimers of type I and type II receptors, followed by phosphorylation of SMAD1, SMAD5 or SMAD8, and translocation of SMAD complexes to the nucleus to activate transcription. This signaling pathway can be blocked by extracellular antagonists, decoy receptors, or intracellular inhibitors.
<table>
<thead>
<tr>
<th>Gene</th>
<th>BMP Inhibitor Name</th>
<th>Known specificity</th>
<th>Family</th>
<th>Known Functions</th>
<th>First description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER1</td>
<td>Cerberus</td>
<td>BMP4, Wnt, Nodal</td>
<td>Can (8-membered)</td>
<td>Spemann organizer; head inducing</td>
<td>Bouwmeester, 1996 [167]</td>
</tr>
<tr>
<td>DAND5</td>
<td>Coco</td>
<td>BMP4, TGFβ, Wnt</td>
<td>Can (8-membered)</td>
<td>Cell fate decision during gastrulation; breast cancer metastasis to lung</td>
<td>Bell, 2003 [168]</td>
</tr>
<tr>
<td>NBL1</td>
<td>Dan</td>
<td>BMP2, BMP4, GDF5, Slit1, Slit2</td>
<td>Can (8-membered)</td>
<td>Downregulated in transformed fibroblasts, expressed in axon tracts</td>
<td>Stanley, 1998 [169]</td>
</tr>
<tr>
<td>GREM1</td>
<td>Gremlin</td>
<td>BMP2, BMP4, BMP7, Slit1, Slit2, VEGFR2</td>
<td>Can (8-membered)</td>
<td>Kidney, lung, limb development; expressed by fibroblasts and endothelium in cancer</td>
<td>Hsu, 1998 [170]</td>
</tr>
<tr>
<td>GREM2</td>
<td>Gremlin2 / PRDC</td>
<td>BMP2, BMP4</td>
<td>Can (8-membered)</td>
<td>Ovary, brain, spleen, follicle development</td>
<td>Minabe-Saegusa, 1998 [171]</td>
</tr>
<tr>
<td>SOST</td>
<td>Sclerostin</td>
<td>BMP2, BMP4, BMP6, BMP7</td>
<td>Can (8-membered)</td>
<td>Specific to bone, bone development</td>
<td>Kusu, 2003 [172]</td>
</tr>
<tr>
<td>SOSTDC1</td>
<td>SOSTDC / USAG-1 / Wise</td>
<td>BMP2, BMP4, BMP6, BMP7, Wnt3a, LRP5, LRP6</td>
<td>Can (8-membered)</td>
<td>Kidney; expressed in brain ventricles, teeth; sensitizes endometrium for implantation</td>
<td>Simmons, 2002 [173]</td>
</tr>
<tr>
<td>TWSG1</td>
<td>Twisted Gastrulation</td>
<td>BMP2, BMP4</td>
<td>Tsg (9-membered)</td>
<td>Works along with chordin, can also enhance BMP signaling by promoting chordin degradation; Dorsal fates in drosophila</td>
<td>Mason, 1994 [174]</td>
</tr>
<tr>
<td>NOG</td>
<td>Noggin</td>
<td>BMP2, BMP4, BMP5, BMP6, BMP7, GDF5, GDF6</td>
<td>Noggin (10-membered)</td>
<td>Spemann organizer, skeletal development, hair follicles, neurogenesis, cancer</td>
<td>Smith, 1992 [175]</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Function</td>
<td>Authors</td>
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<tr>
<td>BMPER</td>
<td>Crossveinless2 / BMPER</td>
<td>BMP2, BMP4</td>
<td>Chordin (10-membered)</td>
<td>Bone, eye, kidney development; neural crest induction</td>
<td>Conley, 2000 [176]</td>
</tr>
<tr>
<td>CHRD</td>
<td>Chordin</td>
<td>BMP2, BMP4</td>
<td>Chordin (10-membered)</td>
<td>Spemann organizer, DiGeorge-like phenotypes, ovarian cancer</td>
<td>Sasai, 1994 [177]</td>
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<td>CHRDL1</td>
<td>Chordin-like1 / Ventroptin</td>
<td>BMP4</td>
<td>Chordin (10-membered)</td>
<td>Angiogenesis, retina</td>
<td>Nakayama, 2001, Sakuta, 2001 [178, 179]</td>
</tr>
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<td>CHRDL2</td>
<td>Chordin-like2</td>
<td>BMP2, BMP4, BMP6, BMP7</td>
<td>Chordin (10-membered)</td>
<td>Patterning, chondrogenesis, expressed in uterus</td>
<td>Nakayama, 2004 [180]</td>
</tr>
<tr>
<td>TSKU</td>
<td>Tsukushi</td>
<td></td>
<td>Chordin (10-membered)</td>
<td>Works in association with chordin in germ layer development</td>
<td>Ohta, 2004 [181]</td>
</tr>
<tr>
<td>VWC2</td>
<td>Brorin</td>
<td>BMP2, BMP6</td>
<td>Chordin (10-membered)</td>
<td>Neurogenesis</td>
<td>Koike, 2007 [182]</td>
</tr>
<tr>
<td>VWC2L</td>
<td>Brorin-like</td>
<td>BMP2, BMP6</td>
<td>Chordin (10-membered)</td>
<td>Neurogenesis</td>
<td>Miwa, 2009 [183]</td>
</tr>
<tr>
<td>FST</td>
<td>Follistatin</td>
<td>BMP4, BMP5, BMP6, BMP7, BMP15, FSH, Activin, TGFβ</td>
<td>Follistatin (10-membered)</td>
<td>Folliculogenesis in the ovary</td>
<td>Fainsod, 1997 [184]</td>
</tr>
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<td>FSTL1</td>
<td>Follistatin-like1</td>
<td>BMP2, Activin</td>
<td>Follistatin (10-membered)</td>
<td>Immunoregulation</td>
<td>Tsuchida, 2000 [185]</td>
</tr>
<tr>
<td>FSTL2</td>
<td>Follistatin-like2</td>
<td>BMP7</td>
<td>Follistatin (10-membered)</td>
<td>Spemann organizer</td>
<td>Dal-Pra, 2006 [186]</td>
</tr>
<tr>
<td>Cancer Type</td>
<td>Citation</td>
<td>BMPs studied</td>
<td>Results</td>
<td>Pro- or Anti-tumorigenic</td>
<td></td>
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<tr>
<td>Bladder Cancer</td>
<td>Kim et al. 2004 [187]</td>
<td>BMP4</td>
<td>BMPR2 is lost in transitional cell carcinoma; reexpression causes decreased tumorigenicity in vivo in response to BMP4</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Breast Cancer</td>
<td>Ren et al. 2014 [188]</td>
<td>BMP9</td>
<td>BMP9 inhibits bone metastases of breast cancer</td>
<td>Anti</td>
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<tr>
<td>Breast Cancer</td>
<td>Ampuja et al. 2013 [189]</td>
<td>BMP4</td>
<td>BMP4 inhibits growth but promotes migration in breast cancer cells</td>
<td>Both</td>
<td></td>
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<tr>
<td>Breast Cancer</td>
<td>Hu et al. 2013 [190]</td>
<td>BMP6</td>
<td>BMP6 inhibits proliferation in breast cancer by targeting miR-192</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Breast Cancer</td>
<td>Owens et al. 2013 [191]</td>
<td>BMP4</td>
<td>BMP4 stimulates mammary carcinoma-associated fibroblasts to secrete proinvasive factors</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Breast Cancer</td>
<td>Lian et al. 2013 [192]</td>
<td>BMP6</td>
<td>Downregulation of BMP6 increases breast cancer cell proliferation and chemoresistance</td>
<td>Anti</td>
<td></td>
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<td>Breast Cancer</td>
<td>Chen et al. 2012 [193]</td>
<td>BMP2</td>
<td>BMP2 inhibits the proliferation of breast cancer cells via p21 and caspase induction</td>
<td>Anti</td>
<td></td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>Buijs et al. 2011 [146]</td>
<td>BMP2, BMP7</td>
<td>The BMP2/7 heterodimer decreases the CSC population in breast cancer and decreases bone metastases</td>
<td>Anti</td>
<td></td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>Montesano et al. 2008 [140]</td>
<td>BMP4</td>
<td>BMP4 enhances proliferation of breast cancer cells in the presence of growth factors</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Breast Cancer</td>
<td>Montesano et al. 2007 [142]</td>
<td>BMP4</td>
<td>BMP4 promotes invasive growth of breast cancer cells</td>
<td>Pro</td>
<td></td>
</tr>
<tr>
<td>Cancer Type</td>
<td>Authors</td>
<td>Target</td>
<td>Effect Description</td>
<td>Pro/Anti</td>
<td></td>
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<tr>
<td>Breast Cancer</td>
<td>Clement et al. 2005 [141]</td>
<td>BMP2</td>
<td>BMP2 increases the invasive phenotype and hormone-independent growth of breast cancer cells</td>
<td>Pro</td>
<td></td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>Pouliot et al. 2002 [139]</td>
<td>BMP2</td>
<td>BMP2 inhibits the proliferation of breast cancer cells via p21 induction</td>
<td>Anti</td>
<td></td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>Lombardo et al. 2011 [149]</td>
<td>BMP4</td>
<td>BMP4 causes differentiation in colorectal cancer stem cells and promotes sensitivity to chemotherapy</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Colon Cancer</td>
<td>Beck et al. 2007 [195]</td>
<td>BMP2</td>
<td>Prolonged BMP treatments in colon cancer caused an increase in growth due to decreases in PTEN expression</td>
<td>Pro</td>
<td></td>
</tr>
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<td>Colon Cancer</td>
<td>Beck et al. 2007 [196]</td>
<td>BMP2</td>
<td>BMP2 decreases proliferation in colon cancer via p21 induction</td>
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<td>Colon Cancer</td>
<td>Beck et al. 2006 [197]</td>
<td>BMP2, BMP7</td>
<td>BMP2 and BMP7 are expressed and decrease proliferation in colon cancer via a SMAD-independent pathway</td>
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<td>Colon Cancer</td>
<td>Deng et al. 2006 [198]</td>
<td>BMP4</td>
<td>BMP4 is expressed in colon cancer and promotes invasion and resistance to serum-starved apoptosis</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Colon Cancer</td>
<td>Haramis et al. 2004 [199]</td>
<td>BMP4</td>
<td>BMP4 inhibition causes polyp growth similar to juvenile polyposis syndrome</td>
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<td>Esophageal Cancer</td>
<td>Xu et al. 2013 [200]</td>
<td>BMP7</td>
<td>BMP7 is expressed in metastatic esophageal cancer cells and knockdown of BMP7 by siRNA reduced migration/invasion</td>
<td>Pro</td>
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<td>Esophageal Cancer</td>
<td>Rees et al. 2006 [144]</td>
<td>BMP7</td>
<td>BMP7 reverses EMT changes in esophageal adenocarcinoma</td>
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<td>Gastric Cancer</td>
<td>Zhang et al. 2012 [201]</td>
<td>BMP2</td>
<td>BMP2 decreases proliferation of gastric cancer cells by downregulating CDK4</td>
<td>Anti</td>
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<tr>
<td>Gastric Cancer</td>
<td>Kang et al. 2009 [202]</td>
<td>BMP2</td>
<td>BMP2 enhances the mobility of gastric cancer cells via Akt activation</td>
<td>Pro</td>
<td></td>
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<td>Tumor Type</td>
<td>Authors</td>
<td>BMP</td>
<td>Effect</td>
<td>Direction</td>
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<td>GBM</td>
<td>Chirasani et al. 2009</td>
<td>BMP7</td>
<td>NSCs promote differentiation in GBM CSCs by secretion of BMP7</td>
<td>Anti</td>
<td></td>
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<td></td>
<td>Lee et al. 2008</td>
<td>BMP2</td>
<td>Some GBM CSCs express BMPR1a and have a proliferative response to BMP2</td>
<td>Pro</td>
<td></td>
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<tr>
<td></td>
<td>Ciceroni et al. 2008</td>
<td>BMP4</td>
<td>Glutamate receptors maintain GBM CSCs by inhibiting BMP4 signaling</td>
<td>Anti</td>
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<td></td>
<td>Piccirillo et al. 2006</td>
<td>BMP4</td>
<td>BMP4 causes differentiation in GBM CSCs.</td>
<td>Anti</td>
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<td>Hepatocellular Carcinoma</td>
<td>Zheng et al. 2014</td>
<td>BMP2</td>
<td>BMP2 inhibits hepatocellular carcinoma growth and migration by downregulation of Akt</td>
<td>Anti</td>
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<td></td>
<td>He et al. 2014</td>
<td>BMP6</td>
<td>BMP6 is downregulated in hepatocellular carcinoma</td>
<td>Anti</td>
<td></td>
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<td></td>
<td>Herrera et al. 2013</td>
<td>BMP9</td>
<td>BMP9 promotes survival in hepatocellular carcinoma</td>
<td>Pro</td>
<td></td>
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<td></td>
<td>Li et al. 2013</td>
<td>BMP9</td>
<td>BMP9 promotes EMT in hepatocellular carcinoma</td>
<td>Pro</td>
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<td></td>
<td>Zhang et al. 2012</td>
<td>BMP4</td>
<td>BMP4 induces differentiation in hepatocellular CSCs</td>
<td>Anti</td>
<td></td>
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<tr>
<td></td>
<td>Guo et al. 2012</td>
<td>BMP4</td>
<td>Upregulation of BMP4 is associated with poor prognosis in hepatocellular carcinoma</td>
<td>Anti</td>
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<td>Leukemia, Myeloid</td>
<td>Zhao et al. 2013</td>
<td>BMP4</td>
<td>BMP4 prevents apoptosis in AML following chemotherapy</td>
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<td>Lung Cancer, non-small cell</td>
<td>Liu et al. 2012</td>
<td>BMP7</td>
<td>BMP7 increases invasion in lung cancer cells without affecting proliferation</td>
<td>Pro</td>
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<td></td>
<td>Dai et al. 2004</td>
<td>BMP3b</td>
<td>BMP3b is downregulated in NSCLC and its overexpression decreases colony formation</td>
<td>Anti</td>
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<td></td>
<td>Langenfeld et al. 2003</td>
<td>BMP2</td>
<td>BMP2 is overexpressed in NSCLC and stimulates tumor growth</td>
<td>Pro</td>
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<td></td>
<td>Buckley et al. 2003</td>
<td>BMP4</td>
<td>BMP4 induces senescence in NSCLC</td>
<td>Anti</td>
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<td>Tumor Type</td>
<td>Researcher</td>
<td>BMPs</td>
<td>Summary</td>
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<td>Medulloblastoma</td>
<td>Zhao et al. 2008 [136]</td>
<td>BMP2, BMP4</td>
<td>BMP2 and BMP4 decreases proliferation in medulloblastoma via downregulation of Atoh1/Math1</td>
<td>Anti</td>
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<tr>
<td>Medulloblastoma</td>
<td>Iantosca et al. 1999 [214]</td>
<td>BMP2, BMP4</td>
<td>BMP2 and BMP4 inhibit apoptosis in a medulloblastoma cell line</td>
<td>Pro</td>
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<td>Melanoma</td>
<td>Hsu et al. 2008 [157]</td>
<td>BMP7</td>
<td>Melanoma cells secrete Noggin as protection against growth inhibition by BMP7</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Melanoma</td>
<td>Rothhammer et al. 2007 [147]</td>
<td>BMP2, BMP4</td>
<td>BMP2 and BMP4 promote vascular tube formation and angiogenesis in melanoma</td>
<td>Pro</td>
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<tr>
<td>Melanoma</td>
<td>Rothhammer et al. 2005 [215]</td>
<td>BMP4</td>
<td>BMP4 is overexpressed in melanoma and promotes cell invasion</td>
<td>Pro</td>
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<tr>
<td>Multiple Myeloma</td>
<td>Olsen et al. 2014 [216]</td>
<td>BMP9</td>
<td>BMP9 inhibits proliferation of multiple myeloma cells</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Multiple Myeloma</td>
<td>Holien et al. 2011 [217]</td>
<td>BMP4, BMP6</td>
<td>BMP4 and BMP6 induce apoptosis in multiple myeloma cells by downregulation of myc</td>
<td>Anti</td>
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<tr>
<td>Osteosarcoma</td>
<td>Lv et al. 2013 [218]</td>
<td>BMP9</td>
<td>BMP9 decreases invasive potential of osteosarcoma cells</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Osteosarcoma</td>
<td>Wang et al. 2011 [148]</td>
<td>BMP2</td>
<td>BMP2 causes differentiation in osteosarcoma CSCs</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Osteosarcoma</td>
<td>Weiss et al. 2006 [150]</td>
<td>BMP2</td>
<td>BMP2 is more highly expressed in metastatic osteosarcoma cells</td>
<td>Pro</td>
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<tr>
<td>Osteosarcoma</td>
<td>Arihiro et al. 2001 [219]</td>
<td>BMP2, BMP4</td>
<td>BMP2 or BMP4 is expressed more highly in bone metastases of osteosarcoma</td>
<td>Pro</td>
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<td>Ovarian</td>
<td>McLean et al. 2011 [220]</td>
<td>BMP2, BMP4, BMP6</td>
<td>Mesenchymal stem cells promote the CSC population in ovarian carcinoma via altered BMP expression</td>
<td>Pro</td>
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<tr>
<td>Ovarian</td>
<td>Shepherd et al 2007 [221]</td>
<td>BMP4</td>
<td>BMP4 induces the protooncogene ID3 in ovarian cancer</td>
<td>Pro</td>
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<td>Ovarian</td>
<td>Theriault et al. 2007 [222]</td>
<td>BMP4</td>
<td>BMP4 induces EMT and promotes invasion in ovarian cancer cells</td>
<td>Pro</td>
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<td>Tumor Type</td>
<td>Authors et al. Year [Reference]</td>
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<td>Effect/Significance</td>
<td>Pro/ Anti</td>
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<td>Pancreatic Cancer</td>
<td>Liu et al. 2012 [223]</td>
<td>BMP2</td>
<td>BMP2 induces pancreatic cancer cell invasion by induction of MMP2 through reactive oxygen species</td>
<td>Pro</td>
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<td>Prolactinoma</td>
<td>Pereda et al. 2003 [224]</td>
<td>BMP2, BMP4</td>
<td>BMP4 stimulates proliferation of prolactinoma cells via interactions between SMAD proteins and estrogen receptor. BMP2 levels are unchanged between normal and cancer cells.</td>
<td>Pro</td>
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<td>Prostate Cancer</td>
<td>Kwon et al. 2013 [225]</td>
<td>BMP6</td>
<td>BMP6 induces the expression of IL-6 in macrophages, promoting neovascularization</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Prostate Cancer</td>
<td>Lee et al. 2011 [226]</td>
<td>BMP6</td>
<td>BMP6 secreted by macrophages causes neuroendocrine differentiation in prostate cancers, increasing their resistance to therapy</td>
<td>Pro</td>
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<tr>
<td>Prostate Cancer</td>
<td>Ye et al. 2007 [227]</td>
<td>BMP7</td>
<td>BMP7 decreases invasiveness and motility by induction of BMP antagonists</td>
<td>Anti</td>
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<td>Prostate Cancer</td>
<td>Dai et al. 2005 [228]</td>
<td>BMP2, BMP6</td>
<td>BMP2 and BMP6 promote osteoblastic prostate cancer bone metastases</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Prostate Cancer</td>
<td>Feeley et al. 2005 [229]</td>
<td>BMP2, BMP7</td>
<td>BMP2 and BMP7 stimulate the migration and invasion of prostate cancer cells</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Prostate Cancer</td>
<td>Miyazaki et al. 2004 [137]</td>
<td>BMP7</td>
<td>BMP7 decreases proliferation of prostate cancer cells in vivo via p21</td>
<td>Anti</td>
<td></td>
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<tr>
<td>Prostate Cancer</td>
<td>Hamdy et al. 1997 [230]</td>
<td>BMP6</td>
<td>BMP6 is expressed in prostate cancer and increased with metastasis</td>
<td>Pro</td>
<td></td>
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<td>Renal Cell Carcinoma</td>
<td>Lee et al. 2013 [231]</td>
<td>BMP6</td>
<td>BMP6 promotes IL-10-mediated M2 polarization of renal cell carcinoma-associated macrophages</td>
<td>Pro</td>
<td></td>
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<tr>
<td>Thyroid, anaplastic</td>
<td>Franzen et al. 2001 [138]</td>
<td>BMP7</td>
<td>BMP7 causes cell cycle arrest in anaplastic thyroid carcinoma cells via p21 and p27 induction</td>
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Chapter 3

Glioma Stem Cells Secrete Gremlin1 to Promote Their Maintenance Within a Tumor Hierarchy

This chapter is adapted from a manuscript that has been published in *Genes and Development*. This work represents the main focus of my time as a member of Jeremy Rich’s laboratory:


I would like to thank Qiulian Wu and Diana Yan for their help with the knockdown studies in this chapter. In addition, I am extremely grateful for Christine Lee and Nasiha Rahim for their assistance in responding to the reviewer comments for the published studies that I describe here. Lastly, I’d like to thank Isabel Tritschler for her help with the Western Blots in Figure 3.14 and Jennifer DeVecchio for her help with the p53 sequencing in Figure 3.15.
3.1. Abstract

GBMs are the most prevalent and lethal primary brain tumor and are comprised of hierarchies with self-renewing CSCs at the apex. Like NSCs, CSCs reside in functional niches that provide essential cues to maintain the cellular hierarchy. BMPs instruct NSCs to adopt an astrocyte fate and are proposed as anti-CSC therapies to induce differentiation, but paradoxically tumors express high levels of BMPs. Here we demonstrate that the BMP antagonist Gremlin1 is specifically expressed by CSCs as protection from endogenous BMPs. Gremlin1 co-localizes with CSCs in vitro and in vivo. Further, Gremlin1 blocks pro-differentiation effects of BMPs and overexpression of Gremlin1 in non-CSCs decreases their endogenous BMP signaling to promote stem-like features. Consequently, Gremlin1-overexpressing cells display increased growth and tumor formation abilities. Targeting Gremlin1 in CSCs results in impaired growth and self-renewal. Transcriptional profiling demonstrated that Gremlin1 effects were associated with inhibition of p21\(^{WAF1/CIP1}\), a key CSC signaling node. This study establishes CSC-derived Gremlin1 as a driving force in maintaining GBM tumor proliferation and GBM hierarchies through the modulation of endogenous pro-differentiation signals.
3.2. Introduction

Stem cell proliferation is tightly constrained by niches that provide maintenance cues and prevent uncontrolled growth. Upon exiting its niche, a stem cell undergoes loss of self-renewal and lineage specification, thereby linking the microenvironment with the normal cellular hierarchy. Many cancers display similar hierarchies with stem-like neoplastic cells, or CSCs, that maintain tumors and give rise to differentiated progeny [23]. Like their non-transformed relatives, CSCs reside in defined niches but are capable of actively modifying their microenvironment [20, 34].

As explained in previous chapters, GBMs have demonstrated one of the most reliable models of cellular hierarchies with CSCs at the top of the hierarchy and more differentiated progeny (non-stem glioma cells or non-CSCs) below [27]. CSCs are functionally defined by their capacity to self-renew and propagate tumors that phenocopy the parental tumor [23], while non-CSCs are unable to propagate tumors in the same time frame. We and others have shown that CSCs are resistant to current treatment modalities of radiation and chemotherapy. Together, these two properties of tumor formation ability and therapeutic resistance suggest that CSCs might contribute to tumor recurrence and, ultimately, the death of the patient. In addition, CSCs contribute to tumor spread [36], angiogenesis [34], and immune evasion [16, 101], providing a rationale to target CSCs and their supporting niche for therapeutic benefit. Therefore, there have been multiple endeavors to uncover therapies to selectively target the CSC population. A number of potential therapeutic targets have arisen in recent years...
that are expressed by CSCs. These include TGFβ [48, 49, 60, 233, 234], MELK [235, 236], iNOS [82], Ephrins [51, 52], transcription factors such as ASCL1 [67], and Myc [237, 238], and epigenetic modifiers such as MLL [239], among others. Differentiation therapy offers an alternative approach that has been used in hematopoietic cancers with success [3]. However, similar approaches in brain tumors with retinoids and deacetylase inhibitors have had limited efficacy to date [240, 241].

To provide an explanation for the limited efficacy of differentiation-based therapies, it is important to note that despite the growth advantages of CSCs \textit{in vivo}, tumors derived from CSCs rapidly recreate hierarchies with CSCs often representing a minor fraction of the neoplastic compartment. In the previous chapter, I suggest that this occurs because while differentiated cell populations are not strictly responsible for tumor propagation, they may serve as components of the microenvironment to maintain the CSCs, suggesting a potential drive towards continuous creation of a hierarchy. In brain tumors, differentiated tumor cells secrete cytokines such as IL-6 [103] and laminins [104] that stimulate CSC survival and tumor growth. This is similar to a paradigm found in the drosophila testes and human colon, where differentiated progeny provide ligands for stem cell proliferation [105, 106].

Collectively, these observations suggest that the differentiated progeny of CSCs are not “waste” cells as has been hypothesized but rather an essential component of tumors. Therefore, there are likely to be effectors that maintain both CSC and non-CSC components of the tumor hierarchy. Consequently, an
inefficient differentiation therapy will be ineffective against the tumor, as these driving forces will oppose such therapies to re-equilibrate the balance of CSCs and non-CSCs. Targeting these driving forces that maintain tumor hierarchies will offer nodes of fragility that should synergize with current differentiation therapies for therapeutic benefit.

BMP2, BMP4 and BMP7 have been proposed as potential differentiation therapies against GBM that drive astrocytic specification of GBM CSCs [47, 61, 107]. Consequently, BMP-treated CSCs have a decreased proliferation rate and tumor formation in a xenograft mouse model [47], as well as a decreased invasive potential in rat glioma [242]. Recent work has shown that tumor repressive role of BMPs can be modulated through the transcriptional factor Atf3 [62]. BMPs also cause differentiation in colon cancer and osteosarcoma [148, 149]. However, a paradox arises, as a number of groups have demonstrated elevated BMP expression, particularly BMP2, within human gliomas [108, 109]. Further, endogenous NSCs, which demonstrate tropism toward gliomas [243, 244], offer an additional source of BMP7 [61]. Thus, high levels of BMP ligands are present in gliomas despite their apparent negative impact on CSCs. It is hard to rationalize BMPs as a part of the CSC microenvironment, as unchecked BMP signaling throughout gliomas as suggested by these data would theoretically cause rampant differentiation, effectively collapsing the glioma hierarchy.

Here, we resolve this apparent contradiction by investigating the regulation of BMP signaling within human gliomas. Our data show that although
BMP ligands are secreted by both CSCs and non-stem glioma cells, BMP downstream signaling is more active in the non-stem glioma cells. This apparent difference in downstream signaling can be explained by the secretion of the BMP antagonist Gremlin1 by the CSC population. Exogenous Gremlin1 production in non-stem glioma cells promotes CSC-like properties, and targeting Gremlin1 in CSCs impairs their growth. Gremlin1-mediated increases in CSC proliferation can be explained by Gremlin1-mediated downregulation of the cyclin-dependent kinase (CDK) inhibitor p21^{WAF1/CIP1}. Through this mechanism, we establish Gremlin1 as a key player in promoting CSC self-renewal and maintaining the glioma hierarchy in the context of endogenous differentiation signals. Regulation of the BMP-Gremlin1 axis appears to be essential for the tumor hierarchy. Thus, inhibition of Gremlin1 will synergize well with a BMP-based differentiation therapy for GBM treatment.

3.3. Results

**BMP Signaling is Attenuated in CSCs Compared to Non-Stem Glioma Cells**

BMPs have been reported to be highly expressed by human gliomas [108, 109], despite their causing astrocytic differentiation of CSCs and subsequently decreasing their proliferation rates. These data are corroborated by BMP expression in patient tumors in the Repository for Molecular Brain Neoplasia Data (REMBRANDT) dataset (Fig. 3.1). To resolve this apparent paradox, we first determined BMP signaling in different tumor subpopulations.
We isolated functionally validated matched CSC and non-stem glioma cell populations from primary patient specimens and xenografts using the CSC marker, CD133. While CD133 has been controversial as a CSC marker because it has not been universally informative across all tumors and is not likely exclusively expressed by CSCs, we have found in most models tested that acute use of CD133 from an *in vivo* environment can segregate CSCs and non-CSCs. As validation, we interrogated models to be used in this study for the utility of CD133. The validation is presented in Chapter 1. CD133-positive populations represented less than 5% of the cells in our specimens (Fig. 1.4A). CD133-positive populations expressed stem cell markers (Fig. 1.4B-E) and had an increased ability for self-renewal (Fig. 1.4F-K) and tumor formation in immunocompromised mice (Fig. 1.4L-M), supporting CD133 as informative of CSC phenotypes in these models. As a measurement for endogenous BMP pathway activation, we quantified levels of phosphorylated/activated SMAD1, 5 and 8 proteins, the direct downstream mediators of canonical BMP signaling, within matched CSCs and non-CSCs. SMAD phosphorylation and consequent BMP signaling was higher in all non-stem glioma cells tested compared to matched CSCs (Fig. 3.2A). This observation suggests that while BMP signaling is present in gliomas as a whole, the CSC subpopulation possesses a mechanism to inhibit BMP signaling and prevent differentiation.

To investigate the mechanism by which CSCs selectively limit BMP signaling, we first determined the relative levels of pertinent BMP ligands and receptors in CSC and non-CSC fractions. While all tumors expressed BMP
ligands, the relative expression levels of BMP2, BMP4 and BMP7 in CSCs and non-CSCs varied between specimens. Overall, there was no uniform trend in ligand expression that would result in consistent differential BMP signaling (Fig. 3.2B-E). Similarly, we did not detect consistent changes in BMPR1a or BMPR1b receptor expression across tumor specimens to explain the preferential BMP pathway activation (Fig. 3.2B,F,G). Therefore, variations in the levels of BMP ligands or receptors cannot account for the consistent observed differences in BMP pathway activation.

**CSCs Secrete Elevated Levels of the BMP Antagonist Gremlin1**

The BMP pathway is regulated in a stage- and cell-specific fashion by a number of extracellular antagonists [112, 114]. Feedback mechanisms involving simultaneous secretion of both BMPs and antagonists are found in a number of different systems in development and in cancer [122-124, 157]. Therefore, we examined the mRNA expression of several BMP antagonists in CSC and non-stem glioma cell populations. We found robust expression of Gremlin1 in the CSCs, with comparatively modest or absent expression of other antagonists (Fig. 3.3A,B). Therefore, we further interrogated the role of Gremlin1 and found a striking elevation of Gremlin1 expression in CSCs compared to non-stem glioma cells in all samples tested (Fig. 3.3C). We confirmed the differences in Gremlin1 protein secretion via ELISA (Fig. 3.3D). There were no consistent differences in the comparative expression of Chordin across tumor populations (Fig. 3.3E).
observation suggested Gremlin1 production as a mechanism by which CSCs protect themselves from BMPs within the tumor.

To further confirm that Gremlin1 is secreted in a CSC-specific manner, we evaluated Gremlin1 levels both in vitro and in vivo via immunofluorescent staining of bulk tumor neurospheres in cell culture and xenografted and primary patient tumor specimens. We sought to examine Gremlin1 in the context of both stem and differentiation markers. Therefore, we co-stained with CSC markers Sox2, Olig2, Nestin and CD133, oligodendrocyte precursor markers NG2 and O4, endothelial marker CD31, and differentiation markers GFAP, Map2, Tuj1 and PLP (Fig. 3.4). In three xenografted tumors and a primary patient specimen, Gremlin1 was expressed on cells that were also positive for Sox2 and Olig2 (Fig. 3.4A-B). Furthermore, in these same xenografts, Gremlin1 co-stained with stem markers Nestin and CD133, as well as oligodendrocyte precursor markers NG2 and O4 (Fig. 3.4C-F). These observations suggest CSC-specific secretion of Gremlin1. Gremlin1 also co-stained with Sox2 and Olig2 in cultured neurospheres (Fig. 3.5)

In addition, Gremlin1 staining did not co-localize with endothelial cells but was found on cells surrounding CD31-positive tumor vasculature (Fig. 3.6A), a well-characterized niche for CSCs [245]. GFAP and Gremlin1 also had a mutually exclusive staining pattern, consistent with Gremlin1’s role to limit BMP signaling (Fig. 3.6B). Gremlin1 displayed limited co-staining with neuronal markers Map2 and Tuj1, suggesting a BMP-independent mechanism for neuronal differentiation (Fig. 3.6C-D). Finally, the differentiated oligodendroglial
marker, PLP, did not stain our xenograft specimens despite potent staining in normal brain implying the lack of a mature oligodendrocyte lineage in our tumors (Fig. 3.6E).

**Gremlin1 Antagonizes the Functions of BMP in CSCs**

To validate an intact BMP signaling pathway in CSCs and that Gremlin1 could inhibit the effects of BMP ligands, we treated CSCs with exogenous recombinant human BMP2 in conjunction with Gremlin1. By measuring SMAD1, SMAD5, and SMAD8 protein phosphorylation, we found that BMP2 elicited canonical signaling in CSCs and that Gremlin1 inhibited SMAD activation (Fig. 3.7A). Gremlin1 was able to block BMP2-mediated increases in the astrocyte marker GFAP (Fig. 3.7B). Furthermore, Gremlin1 maintained or increased the mRNA expression of Olig2, a CSC marker, in the presence of BMP (Fig. 3.7C). A similar maintenance of the stem cell state by Gremlin1 was confirmed at the protein level by immunofluorescent staining. BMPs decreased the protein expression of the stem cell marker Sox2 with a corresponding increase in GFAP, and Gremlin1 was able to block these effects (Fig. 3.7D).

By inducing differentiation in CSCs, BMPs cause a functional decrease in CSC proliferation and tumor growth [47]. We therefore investigated the impact of Gremlin1 on these important CSC phenotypes. Using a cell titer assay in which ATP is a surrogate for cell proliferation, we determined that Gremlin1 was able to attenuate BMP2-mediated growth inhibition (Fig. 3.8A-C). Upon addition of Gremlin1, CSCs continue to proliferate even in the presence of BMP2. In
addition, in *in vitro* limiting dilution assays, we also found that exogenous Gremlin1 blocked BMP2-mediated inhibition of neurosphere formation (Fig. 3.8D-L).

*Exogenous Gremlin1 Expression Promotes a Stem Cell Phenotype*

We subsequently determined whether the addition of Gremlin1 in the absence of exogenous BMPs would promote a stem cell-like phenotype in our cells. As CSCs express high levels of Gremlin1, we transduced non-CSCs with lentiviruses expressing either Gremlin1 or a GFP control under a CMV promoter. We validated Gremlin1 overexpression by immunoblot and RT-PCR (Fig. 3.9A,B). As expected, Gremlin1 overexpression diminished endogenous BMP signaling in these cells, measured by phosphorylated SMAD1/5/8 expression, indicating the Gremlin1 was functional. Next, we examined differentiation and stem cell marker expression by RT-PCR and immunofluorescent staining (Fig. 3.9C-I). Consistent with inhibition of endogenous BMP-mediated astrocyte differentiation, Gremlin1-overexpressing cells exhibited increased expression of stem markers *Sox2* and *Olig2* (Fig. 3.9C,D) and decreased expression of astrocyte markers *GFAP* and *S100B* (Fig. 3.9E,F). Gremlin1 overexpression did not alter the expression of neuronal (*Map2*, *Tuj1*) or oligodendrocyte (GalC) differentiation markers (Fig. 3.9G-I). In examination of immunofluorescent staining, Gremlin1 overexpression also decreased GFAP and increased Sox2 expression (Fig. 3.9J,K).
Consistent with a less differentiated phenotype, cells transduced with Gremlin1 exhibited increased cell proliferation. We quantified this increase using CellTiter-Glo assays (Fig. 3.10A-C). We also noted that several tumors lost contact inhibition and formed spheroidal colonies (data not shown). When plated in an *in vitro* limiting dilution assay, Gremlin1-overexpressing cells promoted the formation of tumor neurospheres (Fig. 3.10D-I). Finally, we implanted equal numbers of GFP and Gremlin1-overexpressing cells into immunocompromised mice. The addition of Gremlin1 was able to decrease tumor latency in a bulk tumor population (Fig. 3.10J), and increase tumor initiation in a non-stem glioma cell enriched population (Fig. 3.10K).

**Gremlin1 Increases CSC Maintenance**

To establish a functional role of endogenous Gremlin1 in CSCs, we knocked down the expression of Gremlin1 in CSCs using lentiviral-based introduction of non-overlapping short hairpin RNAs (shRNAs): one in the Gremlin1 coding sequence (shGrem1_485) and the other in the 3’UTR (shGrem1_2456). We compared the effects of these shRNAs with a control vector expressing a non-targeting control shRNA sequence that is not expressed in the human transcriptome (NT shRNA). Successful knockdown of Gremlin1 was verified by immunoblot (Fig. 3.11A). Knockdown of Gremlin1 in CSCs caused an increase in *GFAP* expression (Fig. 3.11B) and a decrease in CD133 surface marker expression (Fig. 3.11C-E). In addition, knockdown of Gremlin1 caused a decrease in cell proliferation quantified over time (Fig. 3.12A,B). Lastly,
CSCs with Gremlin1 knockdown displayed decreased sphere formation, a surrogate of self-renewal, as measured by an *in vitro* limiting dilution assay (Fig. 3.12C-F). This effect was not due to changes in cell apoptosis as there was no significant difference in Caspase activity (Fig. 3.12G).

We confirmed the impact of targeting Gremlin1 expression in tumor formation latency in an intracranial xenograft model (Fig. 3.13A). Once the final mouse bearing a tumor derived from control CSCs developed neurological signs (day 35), we sacrificed a mouse from one of the shRNA conditions. H&E staining revealed that cells transduced with the non-targeting shRNA formed highly angiogenic tumors (Fig. 3.13B). In contrast, cells transduced with shRNAs against Gremlin1 demonstrated no detectable tumor formation at day 35. Because of the lack of tumor formation at day 35, we could not determine whether the injected cells were expressing Gremlin1 at this stage. However, when tumors finally formed in the mice in the shRNA conditions, we found that the resulting tumors were able to circumvent the Gremlin1 shRNA and re-express Gremlin1 (Fig. 3.13C-D). Finally, to assess whether Gremlin1 is relevant in the clinical realm, we used publicly available survival and expression data from The Cancer Genome Atlas (TCGA) to correlate Gremlin1 expression with patient survival. Patients were segregated into Gremlin-high expressing and Gremlin1-low expressing groups based on whether *Grem1* levels in each patient were higher or lower than the mean *Grem1* expression in the data set. By this algorithm, 174 patients were segregated into a Gremlin1-high group and 352 patients into a Gremlin1-low group. Plotting patient survival by *Grem1*
expression, we determined that higher levels of Gremlin1 expression are correlated with a worse prognosis in patients (Fig. 3.13E).

**Gremlin1 Effects Are Associated with p21 Inhibition**

To determine potential effectors that mediate cell proliferation effects downstream of Gremlin1, we performed a whole transcript microarray in two distinct GBM CSC models comparing effects of the non-targeting control sequence shRNA and two Gremlin1 shRNAs. Ingenuity analysis of gene expression changes between each Gremlin1 shRNA and the corresponding non-targeting shRNA revealed changes in genes consistent with BMP upregulation (data not shown), as well as a number of cell cycle-related genes (Fig. 3.14A). Based on the phenotypic effects detailed above, we suspected that the Gremlin1-BMP axis might be linked to cell cycle progression in CSCs. We used Ingenuity’s upstream analysis tool to determine top transcription factors that might be responsible for the gene expression changes in our data. Across both shRNAs in both tumor specimens, the top transcription factor predicted by the microarray to be affected by Gremlin1 was p21\(^{\text{WAF1/CIP1}}\) (Table 3.1). Several p21-regulated genes were modified in the array in a manner consistent with p21 upregulation by the Gremlin1 shRNAs (Fig. 3.14B).

Cells progress to the S-phase of the cell cycle following an accumulation of cyclin-dependent kinase activity at the end of the G\(_1\) phase. Cell cycle arrest is often mediated by cyclin-dependent kinase inhibitors (CKIs) that inhibit G\(_1\)-specific CDKs. TGF\(\beta\) family members, including BMPs, modulate cell cycle
progression through the Cip/Kip family of CKIs, in which there are three members: p21, p27 and p57 [246]. While p21 is thought to play an oncogenic role through inhibition of apoptosis, its canonical role is as a tumor suppressor that decreases proliferation [247]. In particular, BMPs have been shown to decrease cancer cell proliferation through modulation of p21 levels in a number of systems, including colon [196], breast [193], prostate [137] and thyroid [138]. Therefore, we hypothesized that Gremlin1 is promoting cell cycle progression in CSCs via p21 inhibition.

We confirmed that p21 is acting downstream of Gremlin1 by blotting for its expression via immunoblot following Gremlin1 overexpression and knockdown. Indeed, Gremlin1 knockdown resulted in re-expression of p21 in CSCs (Fig. 3.14C). Interestingly, there was little or no change in p21 RNA expression (Fig. 3.14B). The oncogene c-myc, a reported downstream target of p21 [248], was inhibited by Gremlin1 knockdown (Fig. 3.14C). Meanwhile, levels of the related CKIs, p27 and p57, were unchanged by Gremlin1 knockdown. Furthermore, Gremlin1 overexpression decreased p21 expression in non-stem glioma cell enriched populations, with no changes in p27 and p57 (Fig. 3.14D). Finally, cell cycle analyses in CSCs showed that Gremlin1 shRNAs caused a G1 cell cycle arrest, consistent with p21 activation (Fig. 3.14E). Therefore, our data suggests that Gremlin1 is playing a key role in suppressing p21 expression in CSCs.

Canonically, p21 is a downstream target of the tumor suppressor p53. Therefore, we sought to determine if the effects of Gremlin1 on p21 are p53-dependent. As the TP53 gene is frequently mutated in GBM [10], we performed
p53 staining in our tumor specimens along with parallel sequencing of the TP53 gene. Positive p53 staining represents a mutation resulting in increased protein stability of p53 [249]. In our hands, there was robust staining of p53 for one of our xenografts, IN528, and a point mutation was discovered in this tumor through sequencing (Fig. 3.15A-B). In contrast, tumor models 3691 and 3565 were p53 wild-type. As Gremlin1 knockdown and overexpression have similar effects in these tumors, it is likely that Gremlin1’s effects on p21 are independent of p53 status.

3.4. Discussion

CSCs are defined by their tumor propagation potential and are often resistant to conventional therapies, suggesting that they represent the resistant subpopulation responsible for tumor recurrence following treatment [24, 25]. Many studies consider CSCs in isolation but an essential aspect of the claims of a CSC identity requires that the original tumor contains a hierarchy and that the non-CSCs are derived from the CSCs. While it is clear that CSCs themselves contribute to tumor growth, there is increasing evidence that differentiated progeny promote tumor growth by forming a supportive niche for CSCs. This suggests that within a given tumor, there is a balance of differentiation and self-renewal to continuously generate differentiated progeny while maintaining the CSC population. Disrupting the balance between these forces may represent a potential target for tumor therapy.
BMPs are key ligands that cause differentiation in CSCs with a corresponding decrease in proliferation [47, 61, 107]. Despite this apparent negative effect on CSCs, endogenous BMPs, particularly BMP2, are secreted at high levels within bulk tumors compared to normal brain tissue [108, 109]. While this seems paradoxical, these observations suggest that BMPs might be drivers of differentiation in the context of a balanced tumor hierarchy. Therefore, this chapter has focused on the potential of a parallel driver of self-renewal to prevent the differentiation that would be caused by high endogenous BMP signaling.

BMP antagonists are expressed as counterbalances to modulate endogenous BMPs in both development and cancer. For instance, controlled secretion of BMPs and corresponding antagonists dictate embryonic patterning during gastrulation [122] and trigger termination of limb development [123]. In basal cell carcinoma, Gremlin1 and Follistatin are secreted by cancer-associated fibroblasts to inhibit BMP-mediated decreases in cell proliferation [154]. In melanoma, coordinated Noggin upregulation protects melanoma cells from BMP-mediated growth inhibition [157]. Furthermore, the BMP inhibitor Coco is secreted to generate a permissive niche for lung metastases of breast cancer cells [161]. These studies and others highlight the potential importance of BMP antagonism in general tumor biology.

Here, we find that the BMP inhibitor Gremlin1 is secreted in a CSC-specific manner to promote CSC maintenance, supporting the following model: First, BMPs are secreted to induce CSC differentiation and generate differentiated progeny important for tumor growth. Second, Gremlin1 is secreted
specifically by CSCs to shield CSCs in a BMP-rich environment. Hence, by
modulating the levels of a driver of differentiation and a corresponding
antagonist, gliomas are able to balance the levels of CSCs and differentiated
progeny and thereby maintain the tumor hierarchy.

We also found a differential expression of Noggin in CSCs compared to
non-stem glioma cells. However, Noggin expression levels were significantly
lower than Gremlin1 and shRNA knockdown of Noggin did not alter CSC
proliferation (data not shown). Furthermore, if Noggin and Gremlin1 played a
similar role, we would expect Gremlin1 knockdown to have a less of an effect
due to compensatory Noggin secretion. Therefore, while there might be some
level of compensatory Noggin secretion in CSCs, Gremlin1 still appears to be the
primary antagonist responsible for hierarchy maintenance. Also, Gremlin1
treatments did not significantly influence CSC biology in the absence of
exogenous BMP treatments. We suspect that the lack of an effect was simply
due to the fact that CSCs are already producing substantial amounts of
Gremlin1.

In this study, we also describe a link between Gremlin1 and the CKI, p21.
This is contrary to previous work where Gremlin1 promoted p21 transcription in
medulloblastoma [164]. However, here, Gremlin1 does not affect p21 mRNA
levels and rather decreases p21 protein levels. In NSCs, which are a relatively
quiescent population, p21 promotes self-renewal through a number of different
mechanisms, including the feedback inhibition of BMP2 [250, 251]. Unlike NSCs,
CSC self-renewal is linked to p21 inhibition and cell cycle progression [87, 237].
Furthermore, p21 has been established as a downstream effector of BMPs in a number of cancers. Therefore, Gremlin1 inhibition of p21 in CSCs provides a key mechanism for Gremlin1’s promotion of CSC self-renewal and prevention of differentiation.

Finally, in addition to uncovering the novel role of Gremlin1 in maintaining hierarchies, this study is informative to BMP-based GBM therapies. BMP-based therapies are designed to overwhelm tumors with differentiation signals, thereby collapsing the hierarchy and converting the entire tumor into a more differentiated tumor mass. However, this study raises the possibility that a patient’s CSCs might tune their levels of secreted Gremlin1 in response to BMP-based targeted therapies, simply compensating for the perturbation in BMP levels by increased antagonist secretion. Further, our overexpression studies suggest that Gremlin1 overexpression in non-CSCs antagonizes BMP signaling in these populations as well and promotes a more stem-like phenotype. This suggests that in the context of a BMP-based therapy, compensatory Gremlin1 secretion might revert partially differentiated populations back into CSCs. This complication should be taken into account as BMPs are considered as differentiation cancer therapies.

An alternative avenue for therapy would be to explore combined therapies. Targeting Gremlin1 in conjunction with BMP therapies will push differentiation in CSCs while at the same time destroying their protective mechanisms to resist differentiation. This would then enhance the effectiveness of BMP-based therapies. Our study suggests that such a combined therapy could be used to differentiate CSCs for therapeutic benefit.
3.5. Materials and Methods

Isolation and Tissue Culture of Glioma CSCs and Matched Non-stem Glioma Cells

As described previously [24, 34, 82], cell cultures enriched for or depleted of CSCs were derived from primary patient brain tumor specimens or specimens passaged for seven or fewer passages as xenografts in immunocompromised mice. Tumor specimens were acutely dissociated using a papain-based dissociation kit (Worthington Biochemical) following the manufacturer's protocol. Following dissociation, cells were cultured for at least six hours in Neurobasal media supplemented with B27 without vitamin A (Gibco), L-glutamine, sodium pyruvate (Invitrogen) and 10 ng/mL each of epidermal growth factor (EGF, R&D systems) and basic fibroblast growth factor (bFGF, R&D systems) for surface antigen recovery. After this brief incubation, CD133 antibody-conjugated magnetic beads (Miltenyi Biotec) or allophycocyanin (APC)-coated CD133 antibody (Millipore) were used to label the bulk tumor population. After an hour of antibody labeling on ice, magnetic columns or flow cytometry were then used to segregate CD133+ and CD133- populations. Functional CSC assays were performed immediately after CD133 sorting by differential expression of stem cell markers, in vitro limiting dilution assays, and tumor formation assays. Cultures enriched for CSCs had increased stem cell marker expression and increased abilities for self-renewal and tumor initiation (Fig. 1.4).

All differential RNA and protein expression data was collected from cell populations lysed immediately following magnetic or flow cytometric sorting. For
treatment and knockdown experiments that necessitated extended cell culture, CSCs were cultured in supplemented Neurobasal media and non-CSCs were cultured in DMEM media with 10% FBS briefly to maintain viability after sorting. Media for both CSCs and non-CSCs were switched to Neurobasal media without EGF and FGF prior to performing any treatment, knockdown or overexpression experiments.

*Lentiviral-mediated Overexpression and Knockdown*

An HIV-based plasmid with the Gremlin1 cDNA sequence driven by the CMV promoter (Genecopoeia) and a GFP control were used for Gremlin1 overexpression experiments. An FIV-based plasmid with shRNA clones (Genecopoeia) targeting Gremlin1 (shGrem1_485 – target sequence: 5'-acagtcgcaccatcatca-3'; shGrem1_2456 – target sequence: 5'-gcaactcgagaagctgct-3') and a matched non-targeting control shRNA sequence that is not expressed in the human transcriptome (NT) were used for Gremlin1 knockdown experiments. Plasmids were co-transfected with packaging vectors psPAX2 and pCI-VSVG (Addgene) into 293FT cells using Lipofectamine 2000 (Invitrogen) or a calcium phosphate transfection method to produce virus. Cells were infected at the time of plating. Knockdown and overexpression were confirmed using Western Blot.
**Immunofluorescent Staining**

Cultured cells or 10 µm thick slices of human or xenografted brain tissue were fixed in 4% formaldehyde and stained using the following antibodies: Rabbit anti-Gremlin1 (Santa Cruz), Goat anti-Sox2 (Santa Cruz), Goat anti-Olig2 (R&D systems), Mouse anti-CD31 (DAKO), Rat anti-CD31 (BD), Mouse anti-Nestin (BD), Mouse anti-CD133 (Miltenyi Biotec), Rat anti-GFAP (Invitrogen), Mouse anti-NG2 (Millipore), Mouse ani-Map2 (Sigma), Mouse anti-Tuj1 (Covance), Mouse anti-Sox2 (R&D Systems), as well as Mouse anti-O4 and Rat anti-PLP (generous gifts from the laboratory of Dr. Paul Tesar, Case Western University). Primary antibodies were incubated for 16 hours at 4C, followed by detection by the following secondary antibodies: Alexa 488 goat anti-Rabbit or 488 goat anti-Rat (Invitrogen), 568 goat anti-Mouse, 568 goat anti-Rabbit or 568 donkey anti-Goat (Invitrogen), and 633 goat anti-Rat (Invitrogen). Nuclei were stained using DAPI, and slides were mounted using Fluoromount (Calbiochem). Images were taken using a Leica DM4000 Upright microscope.

**Microarray and Microarray Analysis**

Microarray hybridization and processing was performed at the Case Western Reserve University Genomics Sequencing Core according to the manufacturer’s protocol, using the GeneTitan Multichannel Instrument (Affymetrix, Santa Clara, CA). Biotinylated cDNA fragments were generated from 500 ng of total RNA, and 180 ng of cDNA was hybridized onto the HuGene
2.1 PEG array (Affymetrix, Santa Clara, CA). The HuGene 2.1 array covers over 30,000 coding transcripts and 11,000 long intergenic non-coding transcripts.

Raw intensity values were normalized by Robust Multi-array Average (RMA) analysis as previously described [252] using the Bioconductor oligo R package [253]. Using the raw gene expression values, fold changes for each gene were calculated between each pair of non-targeting and shRNA conditions. The fold changes were analyzed using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood, CA) with the threshold of a >2-fold expression difference. The array data has been deposited into GEO (Accession Number GSE52846).

**In vitro Limiting Dilution Assay**

For the *in vitro* limiting dilution assay, CSCs were plated at 1, 5, 10, 20, 40 and 80 cells per well into a 96-well plate via flow cytometry. Ten days after plating, the numbers of neurospheres found in each well were quantified by manual counting. Extreme limiting dilution assay analyses (ELDAs) were performed on the data as previously described [254].

**In vivo Tumor Initiation Assay**

All animal procedures were performed in accordance with Cleveland Clinic IACUC approved protocols. Animals were housed in a temperature-controlled vivarium with a 14 hour light, 10 hour dark cycle at no more than five animals per cage. Bulk tumor populations (10,000 cells per mouse), non-stem glioma cell
enriched populations (50,000 cells per mouse) or CSCs (10,000 cells per mouse) were injected intracranially into a NOD-SCID immunocompromised mouse model. Mice were monitored over time for the development of neurological signs, such as lethargy, seizures and/or ataxia, at which their brains were removed. For immunofluorescent staining, anesthetized mice were perfused with PBS and 4% paraformaldehyde (PFA) prior to removal of their brains. Their brains were then fixed in 4% PFA for 24-48 hours, sunk in 30% sucrose, cryopreserved in OCT and sectioned for staining. For H&E staining, brains were removed without perfusion, fixed in 4% PFA, paraffin embedded, sectioned and stained. Immunohistochemistry staining for p53 was performed on paraffin-embedded brains using Mouse anti-p53 (Ventana).

Recombinant BMP and Gremlin1 Treatments

CSCs or non-stem glioma cells were treated with 25 ng/mL BMP2 in the presence or absence of 1 µg/mL Gremlin1 (R&D Systems). Cells were plated in Neurobasal media without EGF and FGF prior to performing treatments.

Real-Time PCR

Total RNA was isolated using the RNeasy isolation kit (Qiagen) or Trizol (Invitrogen) and reverse transcribed into cDNA using the qScript cDNA SuperMix (Quanta Biosciences). mRNA was labeled with SYBR Green MasterMix (Applied Biosystems). Sequences of primers used in this study are given in Table 3.2. Levels of mRNA were measured using an ABI-7900 RT-PCR system (Applied
Expression values were normalized to β-Actin or GAPDH. For Figures 3.3A and 3.3B, these expression values were reported. For all other figures, expression values were further normalized to a control population.

Immunoblots and ELISAs

20-40 µg of cell lysate was resolved on commercially available gradient SDS-PAGE gels (BioRad) or homemade 12% SDS-PAGE gels, transferred to polyvinylidenedifluoride membranes (Millipore) and detected using an enhanced chemiluminescence system (Pierce Biotechnology) or fluorescence-based system (IRDye®, Li-Cor). Primary antibodies that were used included Rabbit anti-P-SMAD 1/5/8 (Cell Signaling), Rabbit anti-SMAD1 (Cell Signaling), Goat anti-Gremlin1 (R&D), Mouse anti-p21 (Cell Signaling), Rabbit anti-p27 (Santa Cruz), Rabbit anti-p57 (Santa Cruz), Mouse anti-c-myc (Santa Cruz), Rabbit anti-BMPR1b (Abgent), Rabbit anti-BMP2 (Abcam), Mouse anti-Tubulin (Millipore) and Mouse anti-Actin (Sigma). A Gremlin1 ELISA Kit (USCNK) was used to perform ELISAs on 1 mL of media harvested from 50,000 cells for 24 hours, following the manufacturer’s instructions.

Cell Viability Assay and Caspase Activity Assay

CSCs infected with shRNA-expressing lentivirus or treated with recombinant proteins were plated in 96 wells at 500 cells per well. Cell titers were determined after a number of days using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). Caspase activity was determined 2 days and 3
days following transduction using the Caspase-Glo 3/7 Assay Kit (Promega). Caspase activity was normalized to Cell Titer activity to account for differences in cell number.

Cell Cycle Analysis

CSCs infected with shRNA-expressing lentivirus were fixed two or three days after transduction in 70% ethanol. Fixed cells were labeled in propidium iodide-staining buffer (0.25% Triton in PBS, 25 µg/mL RNAseA, and 10 µg/mL propidium iodide) for one hour. FACS analyses were performed on the FACS Scan Analyzer (BD, San Jose, CA) and ModFit (Verity Software House) analysis was performed to determine cell cycle fractions.

P53 sequencing

Genomic DNA was isolated from 2,000,000 CSCs on the Qiagen BioRobot EZ1 workstation using the Qiagen EZ1 DNA Tissue kit (Qiagen) following the manufacturer’s protocol. DNA was amplified by standard PCR using primers against exon 4-9 of TP53 as listed in Table 3.3, for 30 cycles with an annealing temperature of 55C. Sequencing was carried out on an ABI 3730xl DNA analyzer (Applied Biosystems). Mutations in exons 4-9 were evaluated using Finch TV software (Geospiza, Seattle, WA) and aligned with the complete sequence of the human p53 gene using Clustal W alignment software [255].
Statistical Analyses

Grouped data are presented as mean +/- standard error. The difference between groups was determined by ANOVA or Student’s t-test using GraphPad Prism software. For survival analysis, survival was represented with Kaplan-Meier survival curves and p-values were calculated using a log-rank test.
Figure 3.1. BMP2 and BMP7 are upregulated in patient gliomas. (A-B) Publicly available REMBRANDT expression data demonstrating that BMP2 and BMP7 are upregulated in patient gliomas (Oligo=oligodendroglioma). ***, p<0.001.
Figure 3.2. Despite differential BMP signaling, BMPs and BMP receptors are not consistently differentially expressed in CSC and non-CSC populations. (A) Immunoblot of SMAD1/5/8 phosphorylation in matched CSC/non-stem glioma cell populations. (B) Western Blot of BMP2 and BMPR1b in matched CSC/non-stem glioma cell populations. RT-PCR expression data of (C) BMP2, (D) BMP4, (E) BMP7, (F) BMPR1b and (G) BMPR1a in matched CSC/non-stem glioma cell populations. *, p<0.05; **, p<0.01; ***, p<0.001.
Figure 3.3. Glioma CSCs and non-CSCs differentially express Gremlin1. (A,B) RT-PCR expression data of a panel of BMP antagonists in CSC/non-stem glioma cell populations. (C) RT-PCR expression data of Grem1 in CSC/non-stem glioma cell populations (D) ELISA protein expression data of Gremlin1 in matched CSC/non-stem glioma cell populations. (E) RT-PCR expression data of Chrd in CSC/non-stem gliomas cell populations. *, p<0.05; **, p<0.01; ***, p<0.001.
Figure 3.4. Gremlin1 colocalizes with stem cell markers in glioblastoma. Immunofluorescent staining for Gremlin1 in three patient-derived xenografts and a primary human specimen with CSC markers (A) Sox2, (B) Olig2, (C) Nestin and (D) CD133, and oligodendrocyte precursor markers (E) NG2 and (F) O4. White arrows indicate colocalization. Scale bar: 10 µm.
Figure 3.5. Gremlin1 colocalizes with CSC populations in cultured neurospheres. Immunofluorescent staining for Gremlin1 in cultured xenograft spheres with Sox2 and Olig2. Scale bar: 10µm.
Figure 3.6. Gremlin1 does not colocalize with differentiation markers in glioblastoma. Immunofluorescent staining for Gremlin1 in three patient-derived xenografts and a primary human specimen in conjunction with (A) endothelial marker CD31, (B) astrocyte marker GFAP, neuronal markers (C) Map2 and (D) Tuj1. (E) Immunofluorescent staining for Gremlin1 in conjunction with mature oligodendrocyte marker PLP in three patient-derived xenografts and normal brain. White arrows indicate colocalization; orange arrows indicate disparate staining. Scale bar: 10µm.
Figure 3.7. Exogenous Gremlin1 can block BMP2-mediated differentiation in CSCs. (A) Immunoblot of phosphorylated and total SMAD proteins following half an hour of BMP2 and/or Gremlin1 treatments. RT-PCR for (B) GFAP and (C) Olig2 expression following three days of BMP2 and/or Gremlin1 treatments. (D) Immunofluorescent staining for stem marker Sox2 and astrocyte marker GFAP following six days of BMP2 and/or Gremlin1 treatments. *, p<0.05; **, p<0.01; ***, p<0.001. Scale bar: 10 µm.
Figure 3.8. Exogenous Gremlin1 can block BMP2-mediated growth inhibition and depletion of self-renewal. (A-C) Growth curves following BMP2 and/or Gremlin1 treatments. (D,G,J) Representative images of neurospheres in IN528, 3691 and 3565 CSCs following ten days of BMP2 and/or Gremlin1 treatments. (E,H,K) In vitro limiting dilution assays and (F,I,L) quantification following ten days of BMP2 and/or Gremlin1 treatments. ***, p<0.001.
Figure 3.9. Gremlin1 expression blocks endogenous BMP2 signaling and differentiation. (A) Immunoblot of Gremlin1 and P-SMAD1/5/8 in GFP versus Gremlin1-overexpressing cells in four non-stem glioma cell-enriched models. RT-PCR for (B) Grem1, (C) Sox2, (D) Olig2, (E) GFAP, (F) S100B, (G) Map2, (H) TuJ1, and (I) GalC expression following Gremlin1 overexpression. (J) Staining for Sox2 and GFAP following Gremlin1 overexpression. (K) Quantification of staining in J. *, p<0.05; **, p<0.01; ***, p<0.001. Scale bar: 10µm.
Figure 3.10. Gremlin1 expression promotes proliferation and tumor growth by non-stem glioma cells. (A-C) Growth curves following Gremlin1 overexpression in IN528, 3691 and 3565 non-stem glioma cells. (D-F) \textit{in vitro} limiting dilution assay following ten days of Gremlin1 overexpression in IN528, 3691 and 3565 non-stem glioma cells. (G-I) Quantification of data in D-F. (J) Kaplan-Meier survival curve for immunocompromised mice injected intracranially with GFP or Gremlin1 overexpressing bulk IN528 tumor cells. (K) Kaplan-Meier survival curve for immunocompromised mice injected intracranially with GFP or Gremlin1 overexpressing 3565 non-stem glioma cells. *, p<0.05; **, p<0.01.
Figure 3.11. Gremlin1 knockdown in CSCs increases GFAP expression and decreases CD133 surface marker expression. (A) Immunoblot of Gremlin1 following infection with a non-targeting control shRNA (NT) or shRNAs directed against Gremlin1 (485, 2456) in two CSC models. (B) RT-PCR for GFAP expression following Gremlin1 knockdown. (C) Quantification and (D,E) FACS plots demonstrating a decrease in the CD133+ population following Gremlin1 knockdown in (D) 3691 and (E) IN528 CSCs. *, p<0.05; **, p<0.01.
Figure 3.12. Gremlin1 knockdown inhibits CSC proliferation and self-renewal. (A,B) Growth curves following Gremlin1 knockdown in IN528 and 3691 CSCs. (C,E) In vitro limiting dilution assay following ten days of Gremlin1 knockdown in IN528 and 3691 CSCs. (D,F) Quantification of data in C and E. (G) Relative Caspase activity in 3691 and IN528 CSCs infected with Gremlin1 shRNAs. *, p<0.05.
Figure 3.13. Gremlin1 knockdown inhibits CSC tumor growth and Gremlin1 expression correlates with survival in GBM patients. (A) Kaplan-Meier survival curves for immunocompromised mice injected intracranially with a vector containing a non-targeting sequence control (NT) shRNA or Gremlin1-shRNA infected 3691 CSCs. (B) H&E staining of brains injected with 3691 CSCs expressing an NT shRNA or Gremlin1 shRNA-infected cells. (C,D) Staining for Gremlin1 in tumors derived from NT shRNA or Gremlin1 shRNA-infected 3691 CSCs. (E) Kaplan-Meier curve of patient survival based on publicly available microarray and survival data from The Cancer Genome Atlas. Scale bar: 10µm.
Figure 3.14. Gremlin1 promotes cell cycle progression in CSCs via p21. (A) Ingenuity pathway analysis of pathways upregulated by Gremlin1-shRNA derived gene expression data. (B) Heat map of gene expression changes following Gremlin1 shRNA knockdown in genes previously characterized in the literature as upregulated or downregulated by p21 – red is higher expression, green is lower. (C) Western Blot of Gremlin1 knockdown in two CSC models for p21, p27, p57, c-Myc and Gremlin1. (D) Western blot of Gremlin1 overexpression in three CSC models for Gremlin1, p21, p27 and p57. (E) Cell cycle analysis following Gremlin1 shRNA knockdown in 3691 CSCs.
Figure 3.15. Xenografts used in this study have differing p53 statuses. (A) Sequencing data of exons 4-9 of the TP53 gene indicating that IN528 is p53 mutant, while 3691 and 3565 are likely p53 wild-type. (B) Staining for p53 in tumor specimens confirming that IN528 is p53 mutant (positive staining), while 3691 and 3565 are p53 wild-type.
Table 3.1. Top transcription factors/regulators predicted to be responsible for the global gene expression changes caused by Gremlin1 knockdown.

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<th>Regulator</th>
<th>Activation</th>
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<th>IN528, shGrem1 2456</th>
<th>3691, shGrem1 485</th>
<th>3691, shGrem1 2456</th>
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### Table 3.2. RT-PCR primers used in this study

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### Table 3.3. Primers for TP53 amplification

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Chapter 4
Mediators of Gremlin1 signaling in Cancer Stem Cells

I’d like to thank Christine Lee for her assistance in performing the Wnt immunofluorescence studies in this chapter, and Will Flavahan for his assistance in the conceptualization of the XBP1 data.

4.1. Introduction

In the previous chapter, we established the BMP antagonist Gremlin1 as a modulator of CSC maintenance in the context of endogenous pro-differentiation signals. Through qPCR, ELISA and immunofluorescent staining of in vivo sections, we found that Gremlin1 is secreted specifically by CSC populations. Overexpression of Gremlin1 in non-CSCs promoted self-renewal, as measured by marker expression, and caused a corresponding increase in proliferation. Knockdown of Gremlin1 led to BMP-mediated astrocytic differentiation, as measured by differentiation marker and CD133 surface epitope expression, and a corresponding decrease in proliferation, mediated in part by upregulation of p21 following knockdown.

Here, we further evaluate the upstream mechanisms that might explain CSC-specific secretion of Gremlin1. Furthermore, we examine an additional downstream pathway by which Gremlin1 promotes CSC maintenance. While Gremlin1 in itself is potentially targetable for GBM therapy, examining its upstream and downstream pathways will not only provide additional insights into CSC biology, but might also enhance the ability to target the Gremlin1 pathway in clinical settings.
First, we identify Wnt signaling as a potential downstream mediator of Gremlin1 signaling. Classically, there is significant crosstalk between BMP signaling and Wnt signaling pathways, particularly in the induction of differentiation [256-258]. Specifically, Wnt signaling and BMP signaling have been shown to regulate each other antagonistically. The BMP antagonist Noggin has been shown to indirectly activate Wnt signaling in skin tumorigenesis [158]. Furthermore, a number of CAN family secreted BMP antagonists, of which Gremlin1 is a part of, have been demonstrated to interact directly with Wnt proteins [168, 259, 260]. Gremlin1 itself has also been shown to affect Wnt signaling in bone [261].

In addition, as described in Chapter 1, the Wnt pathway is strongly implicated in CSC maintenance, as Wnt receptors Frizzled4 [44] and LGR5 [63], as well as signaling mediator Dishevelled2 [64], have been linked to CSC biology. Moreover, Wnt pathway activation has been implicated in the pro-tumor phenotypes of proliferation [262-264] and invasion [262], as well as the inhibition of apoptosis [263] and differentiation [44, 64]. Lastly, other CSC mediators such as the MET receptor, FoxM1 and the transcription factor ASCL1 have been shown to promote CSC maintenance by activating Wnt signaling [65-67]. Therefore, by assessing Wnt signaling in Gremlin1 knockdown and overexpression conditions, we determined that Wnt activation is a secondary mechanism by which Gremlin1 promotes CSC maintenance.

Furthermore, in this chapter, we sought to examine the upstream mechanisms that could differentially promote Gremlin1 expression in CSCs over
non-CSCs. We hypothesized that CSC-associated microenvironmental influences might contribute to this differential expression. As described earlier, hypoxia is one microenvironmental factor that constitutes a niche for CSCs [94]. Under hypoxic conditions, HIF2α is preferentially expressed in CSCs over non-CSCs, and hypoxia has been demonstrated to promote stem cell marker expression [83, 95]. Hypoxia has also been shown to prevent BMP-mediated differentiation in CSCs [97]. Furthermore, Gremlin1 has been reported to be induced by hypoxia in both bone and in lung tissues [265, 266]. By examining Gremlin1 mRNA expression following cellular exposure to hypoxia or hypoxia mimetics, we suggest hypoxia as a microenvironmental regulator that induces Gremlin1 expression.

To pinpoint the molecular mechanism behind hypoxic regulation of Gremlin1, we performed a bioinformatics analysis of the Gremlin1 promoter and genes correlated with Gremlin1 expression. Although we were unable to find a hypoxia-response element in the Gremlin1 promoter to suggest direct regulation by HIF proteins, we did identify a binding element for X-box Binding Protein 1 (XBP1), and additionally, XBP1 expression correlated with Gremlin1 expression in patient specimens. XBP1 is a master regulator of the unfolded protein response (UPR), an innate cellular response to the accumulation of misfolded proteins in the endoplasmic reticulum (ER). UPR activation is common in cancer, as microenvironmental stressors such as hypoxia and nutrient deprivation – the same stressors that support CSCs – can lead to protein misfolding in the ER and subsequent UPR induction [267]. Specifically, the UPR has recently been shown
to potentiate hypoxic responses in HeLa cells and neuroblastoma [268, 269], and XBP1 is even thought to interact directly with HIF1α in breast cancer [270]. Therefore, we hypothesized that XBP1 is responsible for Gremlin1 upregulation in CSCs in hypoxia.

In physiologic settings, the UPR can be both pro-survival and pro-apoptotic. It consists of three parallel molecular pathways, each initialized by transmembrane ER signaling components – activating transcription factor 6 (ATF6), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and inositol requiring enzyme 1 (IRE1) (Fig. 4.1) [271, 272]. Together, these three pathways regulate the expression of pro-survival genes that promote proper protein folding, or induce apoptosis in unmitigated ER stress. In the first pathway, ATF6 is activated by proteolytic cleavage and its downstream targets are primarily pro-survival chaperones involved in protein folding – most notably glucose-related protein 78 (Grp78) or BiP, and glucose-related protein 94 (Grp94) [271]. In the second pathway, oligomerized PERK phosphorylates eIF2α, which pauses protein translation by inactivating eIF2. This PERK-mediated translational block is avoided by certain transcription factors, including activating transcription factor 4 (ATF4), which contains an internal ribosomal entry site (IRES) in its 5’ untranslated region [273]. ATF4 can drive C/EBP homologous protein (CHOP), which is involved in induction of apoptosis under unrestrained ER stress. In the third pathway, IRE1 cleaves XBP1 mRNA into an active, spliced form (XBP1s) [274]. Active XBP1s can then initiate a transcriptional response that includes ER chaperones and P58IPK, a feedback
inhibitor of PERK signaling [275]. These published data support the PERK pathway as pro-apoptotic, while the other pathways are generally pro-survival and inhibit the PERK pathway.

In cancer, UPR activation had been generally considered as a mechanism for apoptotic cell death following certain therapies [62, 276-279], and has also been proposed as a radiosensitizer [280]. However, increasing evidence suggests a pro-tumorigenic role for UPR in cancer. Specifically, XBP1 drives tumorigenesis in multiple myeloma, breast, prostate and nasopharyngeal carcinoma [281-284], and is correlated with survival in breast cancer as well [285]. In addition, XBP1 promotes cell survival and growth in gliomas [286, 287]; also IRE1 knockdown sensitizes glioma cells to oncolytic viruses [288]. Grp78, IRE1 and XBP1 splicing are also involved in angiogenesis [283, 289, 290]. Finally, both IRE1 and PERK pathways have been shown to protect against oxidative stress [291, 292].

In addition to its role in cancer, there is an emerging role of the UPR in CSC and normal stem cell biology. While the PERK-mediated stress pathway is associated with differentiation [293], ATF6-induced ER chaperone Grp78 is associated with the stem phenotype, protecting the inner cell mass during mouse embryonic development [294]. Moreover, the UPR plays a role in head and neck as well as breast CSC maintenance [270, 295], as well as CSC-mediated radioresistance and metastasis [296, 297].

From these published data, we hypothesized that the UPR is a cytoprotective response in GBM CSCs, mediated in part through promoting CSC
induction of Gremlin1 in the hypoxic niche. We performed correlative analyses to demonstrate a potential link between the UPR and gremlin1 expression. Then, by inducing the UPR using drug treatments, we established the UPR as an inducer of Gremlin1 and inhibitor of BMP. We next found CSC-specific UPR activation by Western Blot and immunofluorescence. Finally, we showed that the UPR can promote cell growth and decrease patient survival. All in all, this chapter investigated Wnt signaling as a downstream effector of Gremlin1, and the UPR as an upstream promoter of Gremlin1 in the hypoxic niche.

4.2. Results

*Gremlin1 activates downstream Wnt signaling in CSCs*

To investigate Wnt signaling as a downstream mediator of Gremlin1, we first examined Wnt signaling in non-CSCs following Gremlin1 overexpression. Canonical Wnt signaling is characterized by the nuclear localization of active, non-phosphorylated $\beta$-catenin. In the absence of Wnt signals, $\beta$-catenin is phosphorylated and degraded. Therefore, to investigate if Gremlin1 might be maintaining CSCs through downstream Wnt signaling, I assessed $\beta$-catenin cellular localization by immunofluorescent staining. I found that Gremlin1 overexpression increased the nuclear localization of $\beta$-catenin, suggesting that Gremlin1 was promoting Wnt signaling (Fig. 4.2A). Furthermore, treatment with the Wnt inhibitor IWR1 decreased effects of Gremlin1 overexpression on cell growth, implying that CSC growth effects of Gremlin1 might be due in part by activation of Wnt signaling (Fig. 4.2B).
Lastly, I investigated Wnt signaling following Gremlin1 knockdown in CSCs. Gremlin1 knockdown was able to decrease levels of non-phosphorylated (active) β-catenin, further suggesting that Wnt activation might be a downstream mechanism of Gremlin1 secondary to BMP inhibition (Fig. 4.2C).

_Hypoxia induces Gremlin1 expression_

CSC biology is intimately regulated by the microenvironmental condition of hypoxia, with hypoxic conditions inducing CSC features. In order to determine the effects of hypoxia on Gremlin1 secretion, we cultured non-CSCs in hypoxic conditions of 1% oxygen, and, in parallel, exposure to the hypoxia mimetic, desferrioxamine (DFX). DFX mimics hypoxia by chelating iron and preventing the inactivation of HIFs by prolyl hydroxylase. We then assessed Gremlin1 expression by qRT-PCR. We found that Gremlin1 was strongly induced with both DFX and hypoxia, suggesting that the hypoxic niche contributes to the differential expression of Gremlin1 in CSCs (Fig. 4.3).

_Expression of UPR pathway effectors are correlated with Gremlin1 expression_

In order to determine upstream pathway effectors that might regulate Gremlin1 expression under hypoxia, we used publicly available microarray data from TCGA GBM patients to correlate the expression of all available genes on the array with Gremlin1. Surprisingly, we found correlations between Gremlin1 expression and a number of pro-survival UPR effectors downstream of IRE1 and ATF6, particularly XBP1 and its target EDEM1, as well as Grp94 (Fig. 4.4A-C).
We also found negative correlations between Gremlin1 and PERK-mediated pathway effectors such as GLYT1 (data not shown). There were no significant correlations with the related BMP antagonist, Gremlin2 (Fig 4.4D). Therefore, we hypothesized that Gremlin1 expression might be related to activation of the IRE1-XBP1 pathway in the UPR.

We next examined the Gremlin1 promoter for potential binding sequences for UPR effectors. We found a potential XBP1 binding site on the Gremlin1 promoter, 492 base pairs upstream of the Gremlin1 start site (Fig. 4.4E). This binding site was conserved between human and mouse, and was not present on Noggin or Gremlin2 (data not shown). This implied that XBP1 might regulate Gremlin1 at the transcriptional level by directly binding to the Grem1 promoter. We also did not find a hypoxia-response element on the Grem1 promoter, suggesting an absence of direct binding by HIF proteins.

**UPR activation increases Gremlin1 expression and decreases BMP signaling in CSCs**

In order to determine if Gremlin1 might be regulated by the UPR, we used small molecule drugs that induce an unfolded protein response, Thapsigargin and Tunicamycin. Thapsigargin induces the UPR by inhibiting the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) pump, subsequently decreasing calcium stores in the ER [298]. Tunicamycin inhibits GlcNAc phosphotransferase, causing protein misfolding, and is also used as a tool to induce UPR [299]. After treating CSCs with each of these drugs, we found an
induction of Gremlin1 at the RNA level at 6, 12, and 24 hours (Fig. 4.5A-C). We did not see a corresponding increase in Noggin expression (Fig. 4.5D). In addition, we saw a decrease in phosphorylated SMAD1/5/8 following Thapsigargin treatment, consistent with Gremlin1 induction following treatment and a corresponding decrease in BMP signaling (Fig. 4.5E).

Cancer Stem Cells have increased activation of XBP1

To establish the UPR as a pathway responsible for CSC-specific secretion of Gremlin1, we next sought to demonstrate CSC-specific activation of XBP1. Therefore, we acutely sorted for CSCs and non-CSCs using the CSC marker, CD133. We previously interrogated models to be used in this study for the utility of CD133 (Fig. 1.4, above). We found that the spliced, active form of XBP1 was differentially expressed in the CSC population (Fig. 4.6A). In addition, we examined XBP1 expression in an in vivo setting via immunofluorescent staining of xenografted mouse tumors. We found co-localization of XBP1 with the CSC marker Sox2 (Fig. 4.6B).

Exogenous XBP-1 expression increases proliferation in non-CSCs

We subsequently determined if activation of the IRE1 pathway in our cells would promote a CSC-like phenotype. In order to do this, we overexpressed either a GFP control or the active (spliced) form of XBP1, XBP1s, in non-CSC populations (Fig 4.7A). We found that overexpression of spliced XBP1 promoted
proliferation in our cells, suggesting that XBP1 promotes a stem-like phenotype (Fig. 4.7B).

**UPR activation is correlated with GBM patient survival**

Finally, to assess whether UPR activation has clinical relevance, we used publicly available survival and expression data from The Cancer Genome Atlas (TCGA) to correlate the UPR with patient survival. For several genes involved in the UPR, patients were segregated into high-expressing and low expressing groups based on whether gene expression in each patient was higher or lower than the mean expression in the data set. Plotting patient survival by gene expression, we determined that higher levels of EDEM1, Grp94 (HSP90B1) and Grp78 (HSPA5) expression are correlated with a worse prognosis in patients (Fig. 4.8). Therefore, UPR activation, like Gremlin1, is correlated with increased tumorigenicity and worse patient survival.

### 4.3. Discussion

Intratumoral heterogeneity plays a major role in the therapeutic resistance of GBM and other cancers. Resistant subpopulations of cells are responsible for therapeutic resistance and tumor recurrence. In GBM, we and others previously demonstrated the existence of a cellular hierarchy with a CSC population at the apex and differentiated cells below [23]. We suggested a model for maintenance of this hierarchy where BMPs drive CSC differentiation and Gremlin1 promotes CSC maintenance to support the existence of dual populations within the same
tumor. In this model, Gremlin1 is a CSC-specific BMP antagonist that protects CSCs in an autocrine fashion. Therefore, Gremlin1 is a potential target for differentiation-based therapies against GBM.

Here, we expand on this model by offering upstream and downstream mechanisms for Gremlin1 promotion of CSC maintenance. Downstream of Gremlin1, we propose Wnt signaling as an alternative mechanism for CSC maintenance aside from p21 inhibition as previously described. Preliminary data with Gremlin1 knockdown suggests that CSC specific Wnt signaling might be due in part to Gremlin1. However, further studies are needed, using the TCF/LEF Wnt reporter assay and immunofluorescent staining following Gremlin1 knockdown, in order to determine if Gremlin1 is required for Wnt pathway activation in CSCs. If this is the case, then Gremlin1 becomes an even more attractive molecular target, as blocking Gremlin1 will promote p21 induction as well as block Wnt signaling.

CAN family secreted BMP antagonists such as Gremlin1 have been described as Wnt inhibitors in other systems [168, 259, 260]. Here we describe the opposite effect – a novel role of Gremlin1 as an enhancer of Wnt signaling. This is not unprecedented, as certain BMP antagonists have been shown to both enhance and inhibit BMP signaling depending on cellular context [300]. However, the question remains as to whether Gremlin1 is enhancing Wnt signaling by directly interacting with Wnt ligands or receptors, or if Gremlin1’s effect on Wnt signaling is a downstream effect of its interactions with BMPs.
Examining Wnt signaling downstream of BMP treatments would be a good first step to determine if this is a BMP-dependent or BMP-independent effect.

In investigating upstream regulation of Gremlin1, we describe a novel relationship between an element of the CSC microenvironment – hypoxia – and hierarchy maintenance. We also examine the UPR as the link between the environment and the hierarchy. We determined that pro-survival pathways of the UPR are correlated with Gremlin1 expression, and that UPR activation by small molecule treatments will induce Gremlin1. Furthermore, we identify a conserved binding site for XBP1 on the Gremlin1 promoter. Preliminarily, this suggests that UPR activation, particularly XBP1 splicing, might directly regulate Gremlin1 expression in CSCs. Further studies, such as chromatin-immunoprecipitation (CHIP) studies to prove that XBP1 will bind to the Gremlin1 promoter, are required to solidify this relationship. If the relationship is validated, then inhibition of XBP1 represents a novel method of targeting Gremlin1.

In addition, we identify XBP1 as a novel CSC target that is expressed at a higher level in CSCs and promotes tumor growth and patient survival. While the UPR has been demonstrated to promote stem cell maintenance [294] in addition to cancer growth [281], a role of the UPR in the context of the CSC hierarchy has not been described. As CSCs are responsible for therapeutic resistance of GBM, there is a tremendous need in the field to uncover CSC-specific molecular targets. Therefore, with further validation of the pro-tumorigenic role of XBP1, the UPR can represent a novel pathway in GBM that can be targeted for therapeutic benefit.
4.4. Additional Materials and Methods

Hypoxia and Drug treatments

GBM CSC and non-CSC populations were isolated as described in the previous chapter. For hypoxia treatments, non-CSCs were placed into a hypoxic incubator (1% O2) or treated with desferrioxamine (Sigma-Aldrich). For drug treatments, CSCs or non-stem glioma cells were treated with 10µM of the Wnt inhibitor IWR1 (Sigma-Aldrich), 1 µM Thapsigargin (Sigma-Aldrich), or 2 µM Tunicamycin (MP Biomedicals).

Lentiviral-mediated Overexpression of XBP1 and Knockdown

An HIV-based plasmid with the XBP1 cDNA sequence attached to a GFP tag driven by the CMV promoter (Genecopoiea) and a GFP control were used for XBP1 overexpression experiments. As described earlier, plasmids were co-transfected with packaging vectors psPAX2 and pCI-VSVG (Addgene) into 293FT cells using Lipofectamine 2000 (Invitrogen) or a calcium phosphate transfection method to produce virus. Cells were infected at the time of plating. Overexpression was confirmed using qPCR using the following primers specific to XBP1: Forward 5'-TGCTGAGTCCGCAGCAGGTG-3'; Reverse 3'-GCTGGCAGGCTCTGGGAAG-5'.
**Immunoblots**

ImmunobLOTS were performed as previously described. The antibodies used to detect XBP1 were Rabbit anti-XBP1 (Santa Cruz) and Rabbit anti-XBP1s (Biolegend).

**Immunofluorescent Staining**

Cells or xenografted brain tissues were fixed in 4% formaldehyde and stained using the following antibodies: Rabbit anti-XBP1 (Santa Cruz), Goat anti-Sox2 (Santa Cruz), Rabbit anti-β catenin (Abcam). Primary antibodies were incubated for 16 hours at 4C, followed by detection by the following secondary antibodies: Alexa 568 donkey anti-rabbit, 488 goat anti-rabbit or 568 donkey anti-goat (Invitrogen). Nuclei were stained using DAPI, and slides were mounted using Fluoromount (Calbiochem). Images were taken using a Leica DM4000 Upright microscope.
Figure 4.1. The unfolded protein response.
Figure 4.2. Gremlin1 promotes Wnt signaling in CSCs. (A) Immunofluorescent staining for β-catenin (green) and DAPI (blue) in Gremlin1-overexpressing non-CSCs. (B) Growth curve comparing GFP and Gremlin1-overexpressing non-CSCs in the context of Wnt inhibition with IWR1. (C) Western Blot of total and non-phospho β-catenin following Gremlin1 knockdown. **, p<0.01.
Figure 4.3. Gremlin1 is induced by hypoxia. *Grem1* and *Nanog* mRNA expression in non-CSCs following 3 days of culture in 21% oxygen, 1% oxygen, and under the hypoxia mimic DFX. *** p<0.001.
Figure 4.4. Gremlin1 is correlated with UPR effectors in GBM patients. Gene correlation data of Gremlin1 with UPR effectors (A) EDEM1 (B) HSP90B1 (Grp94) and (C) XBP1 in patients based on TCGA GBM data. (D) Gene correlation data of Gremlin2 and EDEM1 in the same patients. (E) The Gremlin1 promoter in both human and mouse has a binding site for XBP1.
Figure 4.5. Gremlin1 expression increases and BMP signaling decreases with UPR induction. RT-PCR of Gremlin1 expression following (A) Thapsigargin treatments and (B) Tunicamycin treatments in IN528 non-CSCs. (C) RT-PCR of Gremlin1 expression following Thapsigargin treatments in 3691 non-CSCs. (D) RT-PCR of Nog expression following Thapsigargin treatments in 3691 non-CSCs. (E) Western blot of XBP1 splicing and SMAD phosphorylation following UPR induction by Thapsigargin and Tunicamycin and inhibition by cycloheximide.

* p<0.05, ** p<0.01.
Figure 4.6. XBP1 is differentially expressed by CSCs. (A) Immunoblot of XBP1s expression in matched CSCs and non-CSCs. (B) Immunofluorescent staining of XBP1 in conjunction with Sox2 in a xenografted tumor specimen.
Figure 4.7. XBP1s promotes proliferation in non-CSCs. (A) RT-PCR demonstrating XBP-1 overexpression in 3565 and 3691 non-CSCs. (B) Growth curve of 3565 non-CSCs following XBP1 overexpression. **, p<0.01.
Figure 4.8. UPR activation informs patient survival. Kaplan-Meier curves of patient survival based on publicly available microarray and survival data from The Cancer Genome Atlas, segregated by expression of (A) EDEM1, (B) HSP90B1 (Grp94) and (C) HSPA5 (Grp78).
Chapter 5
Conclusions and Future Work

5.1. Conclusions

Cancer is one of the leading causes of death in the industrialized world, and many cancers have evaded our efforts at treatments for decades. GBM, in particular, has a 15 month survival rate, a number that has remained largely unchanged for nearly half a century [7]. Inter- and intratumoral cellular heterogeneity provides an explanation for treatment failure. Intertumoral heterogeneity describes treatment resistance in individuals within populations, while intratumoral heterogeneity describes this same resistance in individual cells with a tumor, thereby providing an explanation for tumor recurrence. My thesis work is an in depth examination into the intratumoral heterogeneity of GBM.

Aside from the inherent cellular heterogeneity provided by the normal tissue where the tumor resides, there is heterogeneity among the tumor cells themselves. One model for the heterogeneity among tumor cells is the hierarchical model, which describes the existence of a tumor hierarchy in GBM with CSCs at the peak and differentiated cells below [23]. In this model, CSCs are tumor-initiating cells responsible for therapeutic resistance and tumor recurrence; however, differentiated cells provide niche signals that maintain the CSC population. With this in mind, I examined the signals involved in supporting both CSCs and differentiated non-CSCs at the same time; thus maintaining both the stem cells and their niche.
To determine the signals involved in hierarchy maintenance, I examined BMP signaling. BMP signaling is involved in both neural development and cancer, and BMPs cause differentiation in GBM CSCs [47] despite being expressed endogenously within GBM [108, 109]. Therefore, I hypothesized that BMPs are one component of a dual signaling mechanism that could be fine-tuned to adjust the relative level of differentiation and self-renewal in GBM to maintain both CSC and non-CSC populations. To find the other component of this mechanism, I turned to extracellular BMP antagonism. BMPs-antagonist loops are involved in processes in development and in cancer, including limb, kidney and germ layer development [122-124], and in prostate cancer and melanoma [157, 159]. In GBM, I identify Gremlin1 as this potential antagonist. This model is presented in Figure 5.1.

In the studies described above, I have established Gremlin1 as a CSC-specific BMP antagonist. Through overexpression and knockdown studies, I find that Gremlin1 blocks exogenous and endogenous BMP-mediated differentiation, thereby promoting cellular proliferation and tumor growth. Further downstream, I determine that Gremlin1 might be affecting CSC phenotypes by both p21 inhibition and Wnt activation. In addition, I implicate the UPR effector XBP1 as an upstream regulator responsible for CSC-specific Gremlin1 induction. My work proposes that Gremlin1, and potentially XBP1 as well, are viable targets against CSCs in GBM. However, there are still many questions unanswered and potential studies to be performed. I propose a few of these studies here.
5.2. Further Studies for Gremlin1 Regulation

In this thesis, I propose Wnt signaling as a potential downstream mechanism for CSC maintenance by Gremlin1. However, these data are still preliminary. In order to show that Gremlin1 maintains CSCs through Wnt signaling, I would like to establish a stronger link between Gremlin1 and Wnt signaling, assessing Wnt activity following Gremlin1 overexpression and knockdown using a Wnt reporter, immunofluorescence, and examination of Wnt target genes. In addition, I would like to perform a number of experiments to investigate tumor phenotypes following Wnt inhibition in the context of Gremlin1 overexpression. These include differentiation assays, sphere formation assays and \textit{in vivo} studies following Wnt inhibition in Gremlin1-overexpressing conditions. Last, I propose the converse experiment – knocking down Gremlin1 and determining if the effects of Gremlin1 knockdown may be rescued by Wnt pathway activation.

The thesis also calls to attention the idea that GBM CSCs might be using dysregulated UPR signaling pathways for maintenance of the tumor hierarchy, by XBP1 activation of Gremlin1. I show that XBP1 transcription correlates with Gremlin1 transcription, and that activating UPR using small molecule drugs will induce Gremlin1 and block BMP signaling. However, there are still studies that need to be completed. First, in order to prove a direct link between XBP1 and Gremlin1 expression, I would like to overexpress XBP1 in non-stem glioma cells and knock down XBP1 in CSCs, and then examine the effects on Gremlin1 expression by RT-PCR in addition to Western Blots or ELISAs. In addition, I
would like to perform CHIP analyses with XBP1 and Gremlin1 to demonstrate that XBP1 does bind to the Gremlin1 promoter. Last, I would like to perform a rescue experiment to show that Gremlin1 knockdown will ablate the protumorigenic effects of XBP1 expression.

Furthermore, activation of XBP1 in GBM CSC populations is a novel observation. Therefore, there are a number of specific studies that need to be done to establish the UPR as a relevant, targetable signaling pathway in CSCs. In this thesis, I used Western blots and immunofluorescent staining to demonstrate CSC-specific expression of active XBP1. In addition, I used an XBP1 overexpression construct to show that XBP1 has effects on non-CSC proliferation. To extend these studies, I would like to examine differentiation and stem marker expression with XBP1 overexpression to determine the effects of XBP1 on differentiation status. In addition, I would like to assess XBP1 effects on self-renewal using sphere formation assays, and to inject XBP1-overexpressing cells in an in vivo mouse model and assess the effect of overexpression on tumor growth. Next, I’d like to repeat these experiments using XBP1 knockdown to establish an importance of endogenous XBP1. Finally, to link XBP1 to hypoxia, I can examine the levels of XBP1 and XBP1s in hypoxic conditions or following DFX treatment.

While I expect the effects of XBP1 in CSCs to be mediated by Gremlin1, there is a distinct possibility that XBP1 might promote CSC maintenance through an alternate mechanism. Also, although preliminary experiments suggest otherwise, there is still the possibility that XBP1 might have negative effects on
CSCs instead. If further efforts to link XBP1 to Gremlin1 fail, this definitely does not preclude XBP1 as a potential therapeutic target (or treatment modality) in CSCs. XBP1 has been shown to promote survival through JNK activation and by enhancement of hypoxic signaling, for example [270, 287]. To investigate other potential pathways, a microarray or RNAseq can be performed with XBP1 overexpression and/or knockdown. Alternatively, if XBP1 is found to affect BMP signaling without impacting Gremlin1, a targeted RT-PCR screen can be performed, examining BMP pathway elements or the antagonists provided in Table 2.2.

5.3. BMPs in Other Cancer Hierarchies

In addition to GBM, similar tumor hierarchies peaked by CSC populations have been described in a number of different tumor types, including colon cancer, breast cancer, melanoma, and osteosarcoma [26, 28, 29, 31, 33]. Therefore, a question that arises is the applicability of the work described here to other cancer types. The idea of non-stem tumor cells providing a supportive niche for CSCs has not been reported outside of GBM. However, reports of CSC isolation have frequently identified CSCs as a minority population, suggesting that there is a drive for maintenance of differentiated cell populations in other tumors. Therefore, it is not unreasonable that similar feedback loops exist in other cancer types.

In colon cancer and osteosarcoma [47, 148, 149], BMPs have been shown to cause differentiation in CSC populations. In colon cancer, the relevant
BMP family member involved is BMP4. An analysis of BMP4 expression in colon cancer shows that BMP4 is upregulated in colon cancer compared to normal colon (Fig. 5.2A). Therefore, similar to GBMs, colon cancers express BMP4 despite its ability to cause differentiation in colon CSCs. These observations set the stage for a similar relationship in colon to what I discovered in GBM – a loop involving a BMP and an antagonist (potentially even Gremlin1) might be involved in colon CSC and niche maintenance. Consequently, following these observations, I examined BMP antagonist expression in colon CSCs vs. non-CSCs from microarray data from Vermeulen and colleagues [301]. I discovered differential expression of a number of BMP antagonists (Fig. 5.2B), which might play a role in a feedback loop similar to GBM.

5.4. Therapeutic Targeting of Gremlin1

Last, the experiments described in this thesis lay caution toward BMP-based differentiation therapies, while at the same time setting the stage for potential therapeutic targeting of Gremlin1. BMPs are currently being pursued as a therapy against GBM, but as described in chapter 3, it is possible that a BMP-based therapy might cause GBMs to recalibrate their relative levels of BMP and antagonist secretion to maintain CSC populations in the context of such therapies. There are two potential ways to circumvent this complication. First, a BMP that is refractory to Gremlin1 inhibition may be used. A variant BMP7 that has been demonstrated to induce CSC differentiation has been described [302],
and preliminary data from another group has indicated that this particular variant is refractory to Gremlin1 inhibition (data not shown).

A second method to improve on current BMP-based therapies is by concurrent inhibition of Gremlin1. Furthermore, since I demonstrated that Gremlin1 might also be acting through Wnt signaling, Gremlin1 might also be a target for monotherapy. As demonstrated by the use of antibodies to target secreted VEGF [303], antibodies may be designed to target Gremlin1 as well. As a preliminary test, I first examined nonmalignant (epileptic) brain tissue for Gremlin1 staining, and found little or no Gremlin1 staining compared to tumor sections, suggesting that Gremlin1 is expressed more highly in GBM compared to normal brain (Fig. 5.3). In addition, by treating CSCs in culture, I have noticed demonstrable effects of anti-Gremlin1 antibodies on tumor cell proliferation, further suggesting this as a possibility (data not shown). Another way to target Gremlin1 would be to use a different BMP variant that can bind Gremlin1 but not signal through BMP receptors. This has been shown to be efficacious in bone – a group has engineered a BMP2 variant to block Noggin in order to promote osteogenesis [304]. Both of these methods will need to be validated both in vitro and in vivo before they are pursued as therapies. However, I hope that in the future, Gremlin1 can be targeted through one of these methods in the presence or absence of concurrent BMP treatments for therapeutic benefit in GBM patients.
Figure 5.1. Project Model. BMP promotes differentiation in CSCs, whereas Gremlin1 promotes self-renewal. The interplay between Gremlin1 and BMP signals maintain both CSCs and non-CSC populations.
Figure 5.2. BMP4 is upregulated in colon cancer, and BMP antagonists are differentially regulated in colon CSCs. (A) Publicly available expression data from The Cancer Genome Atlas demonstrating that BMP4 is upregulated in colon cancer patients versus normal colon. (B) Analysis of gene expression data in colon CSCs and non-CSCs indicating differential expression of BMP antagonists.
Figure 5.3. Gremlin1 is upregulated in GBM. Immunofluorescent staining for Gremlin1 (red) expression in three epileptic brain specimens and one primary GBM.
References


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