

## Therapeutic Potential for Bone Morphogenetic Protein 4 in Human Malignant Glioma



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### Abstract

Human glioma, in particular, malignant forms such as glioblastoma exhibit dismal survival rates despite advances in treatment strategies. A population of glioma cells with stem-like features, glioma cancer stem-like cells (GCSCs), contribute to renewal and maintenance of the tumor cell population and appear responsible for chemotherapeutic and radiation resistance. Bone morphogenetic protein 4 (BMP4), drives differentiation of GCSCs and thus improves therapeutic efficacy. Based on this observation it is imperative that the clinical merits of BMP4 in treating human gliomas should be addressed. This article reviews BMP4 signaling in central nervous system development and in glioma tumorigenesis, and the potential of this molecule as a treatment target in human gliomas. Further work needs to be done to determine if distinct lineages of GCSCs, associated with different glioma sub-classifications, proneural, neural, classical and mesenchymal, differ in responsiveness to BMP4 treatment. Additionally, interaction among BMP4 and cell matrix, tumor-vascular molecules and microglial immune cells also needs to be investigated, as this will enhance our knowledge about the role of BMP4 in human glioma and lead to the identification and/or development of novel therapeutic approaches that improve treatment outcomes of these devastating tumors.

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### Introduction

Brain tumors are the most common cancer with nearly 78,000 new cases including ~25,000 primary malignant brain tumors expected to be diagnosed this year, and ~17,000 people expected to lose their battle with these tumors [1]. Gliomas represent 27% of all brain tumors and 80% of all malignant tumors. Glioblastoma (GBM) accounts for 55.1% of all malignant gliomas with an estimated 12,120 new cases predicted in 2016 [1]. Despite significant advances in elucidating the biological mechanisms of these tumors and even with radical surgical resection followed by adjuvant radiotherapy and/or chemotherapy, patients have poor clinical prognosis with a median survival time of less than 15 months [2], primarily due to tumor recurrence in combination with the development of chemo- and radio-therapeutic resistance [3]. In light of this, novel therapeutic approaches to improve survival in patients with these malignancies must be identified.

Within the past decade, a population of glioma cells with stem-like features, glioma cancer stem-like cells (GCSCs), has been shown to contribute to renewal and maintenance of the tumor cell population. Recent investigations highlight distinct glioblastoma lineages, including proneural, neural, classical and mesenchymal based on transcriptome analyses [4,5], additional work with single-cell RNA-sequencing,

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challenges the clinical relevance of molecule subclasses, as it shows co-existence of multiple sub-classes in the same glioblastoma [6]; heterogeneity most likely reflects glioma cell origin and guides paths of progression. It is hypothesized that GCSCs are responsible for the development of multidrug resistance [3,7] and radioresistance [8–10]. GCSCs share characteristics with neural stem cells (NSCs), including the capacity for self-renewal, multipotency and the ability to form neurospheres in culture [11,12]. GCSCs also express stem cell markers such as CD133, Sox2, Nestin, etc., although tumor heterogeneity places significant limits on the use of markers alone for GCSC identification [7,12–14], even when the cells can be isolated from surgical specimens [15]. Provided that these cells are major culprits in the initiation, development and therapeutic response of gliomas, then targeting and reversing GCSC malignancy is critical to increase therapeutic efficacy and improve patient prognosis.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor *beta* (TGF- $\beta$ ) family, are critical during development and play a role in maintaining adult NSC populations, regulating both self-renewal and differentiation [16–19]. For instance, during development BMP4 induces NSC exit from the cell cycle and differentiation towards an astroglial fate [20]. Increasing evidence indicates that BMP4 is a regulator in human tumorigenesis, including human gliomas. BMP4 can drive GBM cell differentiation, with dysfunction in the BMP pathway inhibiting differentiation of GBM-initiating cells [21]. BMP4 significantly reduces the GCSC population. Transient *in vitro* exposure to BMP4 abolishes the capacity of transplanted GBM cells to establish intracerebral GBM. *In vivo*, delivery of BMP4 effectively blocks tumor growth and associated mortality in mice with intracerebral human GBM cell grafts, through activation of cognate receptors (BMPRs) that trigger the Smad signaling cascade [22]. Although BMP signaling is associated with driving astrocyte and GBM stem cell differentiation, it has also been associated with driving proliferation of early hindbrain progenitors or GBM stem cells [23]. These contrasting effects are context dependent, with the epigenetic state of the cell impacting outcome. For example the BMP4 signaling pathway is activated and results in diffuse intrinsic pontine gliomas (DIPG), a pediatric high grade brainstem glioma, if an H3K27M mutation in H3.1, observed in 80% of DIPGs [24], is present in conjunction with other mutations including Activin Receptor Type1 (AVCR1) or p53, or abnormal signal pathway activation of the PI3K pathway [23–28].

Recent studies have illuminated genetic and epigenetic regulation and roles of BMP4 in brain development, and in malignant brain tumors. Understanding mechanisms of BMP4 regulation, for example, BMP4 expression in GCSCs can be reduced by silencing its promoter, via the action of PRC2, a H3K27 methyltransferase [21,29], and whether and how this molecule brings about its effects in GCSCs derived from distinct glioma lineages, will elucidate mechanisms of tumor development and new treatment opportunities. This review summarizes BMP4 signaling in malignant gliomas and then focuses on genetic and epigenetic aspects of BMP4 signaling as a therapeutic target in malignant glioma development and progression.

### BMP4 Signaling

BMP4, (also known as BMP-2B) was purified and cloned in 1988 [30], with human BMP4 highly conserved [31]. BMP4 signaling is transduced through the canonical TGF- $\beta$  family pathway [32,33], through one of two mechanisms (Figure 1). In the first option, glycosylated BMP4 forms homodimers in the extracellular matrix, and subsequently binds to a preformed complex (PFC) consisting of BMP

Type I serine–threonine kinase receptor BMPRIA or BMPRIB and a Type II receptor, BMPRII, which preferentially activates the canonical signaling mothers against decapentaplegic (SMAD) pathway [34,35]; specifically these complexes preferentially phosphorylate receptor associated SMAD 1, 5 or 8 (known as R-SMADs) [36]. Activated R-SMADs can form heterogenic complexes with co-SMAD4, which translocate to the nucleus, where it acts as a transcription factors (TF) [37]. Alternatively, the BMP4 homodimer may bind to the high affinity Type I receptor which recruits the Type II receptor to the BMP4 induced signal complex (BISC), to preferentially activate the p38/mitogen-activated protein kinase (MAPK) pathway [34,35]. In addition to the canonical pathway, BMP4 may interact with structurally similar receptors, such as Activin Receptor Type 1, Activin Receptor Type II or Type IIB [38–40].

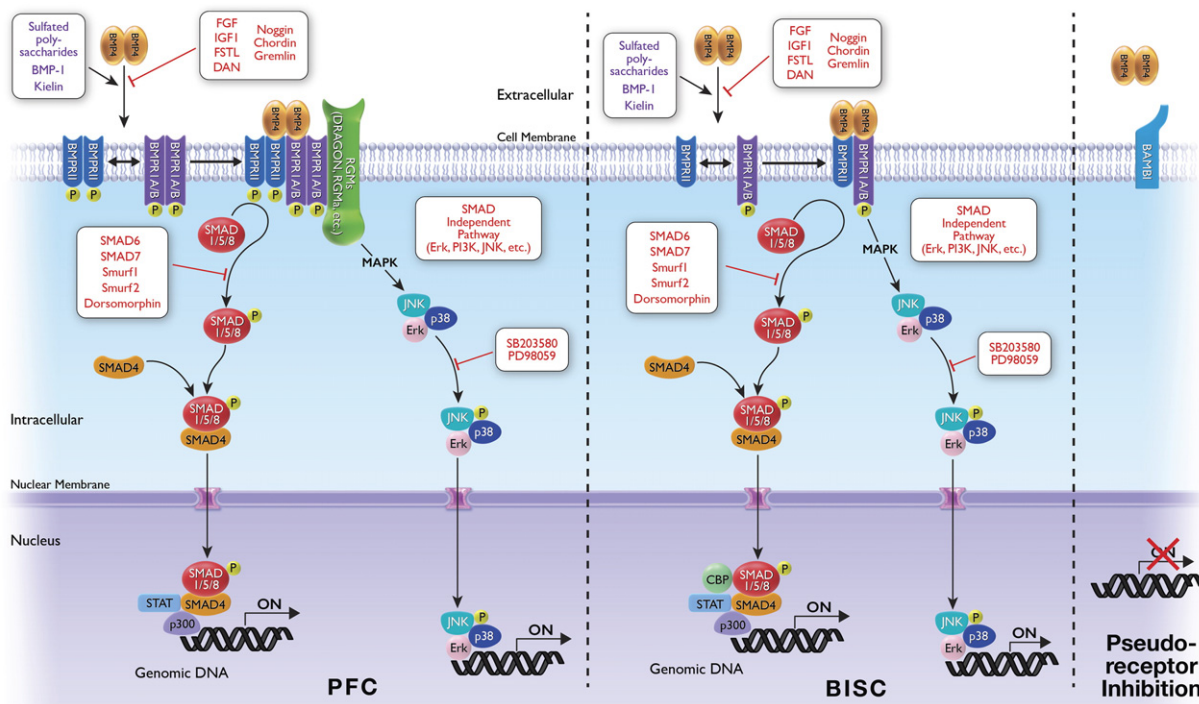
The BMP4 signaling pathway is regulated by numerous extracellular and intracellular factors (Figure 1), as precise spatiotemporal regulation is critical for BMP4 function during development and adulthood. Endogenous extracellular BMP4 activators [41,42] are crucial to balance BMP4 effects on dorsalization during gastrulation [43,44]. Receptor co-binding partners [45–47] inhibit or enhance BMP4 homodimer ligand binding to regulate downstream signaling. Signaling may also be regulated by heterodimer formation between BMP4 and other BMPs, these types of interactions are believed to promote more effective signal transduction [48]. Inhibitors, such as SMAD6 etc., regulate BMP4 signaling at an intracellular level [49–52].

BMP4 regulates downstream gene expression which is dependent on the ligand-receptor combinations at the cellular membrane [36]. Ligand-receptor interaction could be affected by the composition of BMP dimer ligand, whether signaling occurs through the PFC or BISC, and the relative contributions of the two different Type I receptors in mediating downstream activity. For instance, variable signaling through BMPRIA/BMPRII versus BMPRIB/BMPRII complexes occur at different stages in development, affecting unique downstream targets and regulating diverse cellular processes [53,54]. Moreover, a novel non-SMAD protein, PAWS1, associated with SMAD1, may phosphorylate BMPRIA and up-regulate SMAD4 independent target genes, indicating interactions beyond the canonical BMP signaling mechanisms [55].

### BMP4 and CNS Development

BMPs are critical in embryogenesis, including regulation of neural progenitor fate. BMPs function in neural crest [56,57] and hence peripheral nervous system (PNS) development [58,59]; in central nervous system (CNS) development they promote differentiation of neuronal precursors in the spinal cord [60] and cortex [61] and promote astroglial lineage commitment by forebrain subventricular zone (SVZ) progenitor cells [62]. BMPs also induce apoptosis of neural cells including early telencephalic neuroectoderm [63,64] (Figure 2).

Inhibition of BMPs during early embryonic development results in neural induction [65]. Human embryonic stem cells treated with noggin activate microRNA-mediated degradation of SMAD4 transcripts, which may block BMP4-SMAD signaling during neural induction [66]. Repression or activation of BMP in combination with Sonic Hedgehog (Shh) expression specifies ectoderm differentiation into neuronal or non-neuronal tissue, respectively. Molecules, such as FGF and IGF1, also suppress BMP4 at this stage [67,68]. BMP4 and Shh are critical in spinal cord development and establishment of the dorsoventral axis, with the area of intermediate signaling specifying neural crest cells (NCCs) that will form the peripheral sympathetic and sensory nervous systems [69–71] (Figure 2). Deletion of



**Figure 1.** BMP4 cellular signaling pathway including activators and inhibitors. BMP4 may bind to preformed complexes (PFCs), in which, BMPRI Type I and Type II receptors are bound at the cell surface; or to Type I receptors which then complex with Type II receptors to form BMP4 induced signaling complexes (BISCs). Co-binding partners (RGMs, DRAGON, BAMB1, etc.) may enhance BMP4 dimer binding of Type I receptors in both PFCs and BISCs. BMP4 receptor binding activates the canonical SMAD-dependent signal pathway, via phosphorylation of receptor associated SMADs (Smad1/5/8). Activated SMADs form complexes with Co-SMAD4, enter the nucleus and in combination with co-binding partners, such as p300 or STAT, act as transcription factors which drive neural and astroglial gene expression. Extracellular activators such as sulfated polysaccharides, BMP-1, or kielin and inhibitors of BMP4 including Noggin, Chordin, and Gremlin, enhance or inhibit activity of this signal pathway, respectively. Intracellular inhibitors SMAD6/7, Smurf1/2 and dorsomorphin may block pathway activity. BMP4 may also activate SMAD-independent pathways, for instance, MAPK/p38, JNK, Erk etc. Pseudo-receptors such as BAMB1 can bind to BMP4 dimers, however these pseudo-receptor complexes do not activate downstream signaling cascades due to lack of kinase domains.

BMPRIA/B in the neural tube disrupts proper dorsal-ventral interneuron specification. Interactions between BMPs and WNT/ $\beta$ -catenin [72,73] and Notch signaling [74], also regulate NCC specification [58]. For example, shortly after NCC formation, BMP4 and WNT/ $\beta$ -catenin regulate dorsal interneuron specification through transcriptional regulation of neuron-specific TFs including neurogenin-1 (Ngn1) and Olig3 [75]. Following neural induction, secretion of BMP4 from ectoderm and neural tube roof plate cells promotes subsequent neural patterning of several key CNS topographies, including forebrain [76], cerebellum, and dorsal spinal cord [77], as well as early postnatal cerebellar cell differentiation [78].

BMP4 temporally influences neuronal and glial differentiation of embryonic NSCs and neural progenitor cells (NPCs). Following gastrulation, BMP4 signaling, mediated through BMPRIA [61], specifies NSCs and NPCs towards neuronal lineage commitment in the CNS and PNS [79]. Moreover, BMP4 balances NPC quiescence and proliferation to maintain the subgranular zone stem cell population in the adult hippocampus, blocking BMPs with noggin depletes NPCs [80]. BMP4 also promotes differentiation of NSCs or NPCs towards a glial fate, but suppresses differentiation towards a neural or oligodendroglial fate in adult CNS [17,20,81] (Figure 2), with noggin and neurogenesis-1 driving NPC differentiation towards a neural fate [17,81,82].

BMP4 is also a key mediator in the signal network involved in forebrain cortical neurogenesis (Figure 2), inducing rat primary cerebral stem cell neuronal differentiation through SMAD independent

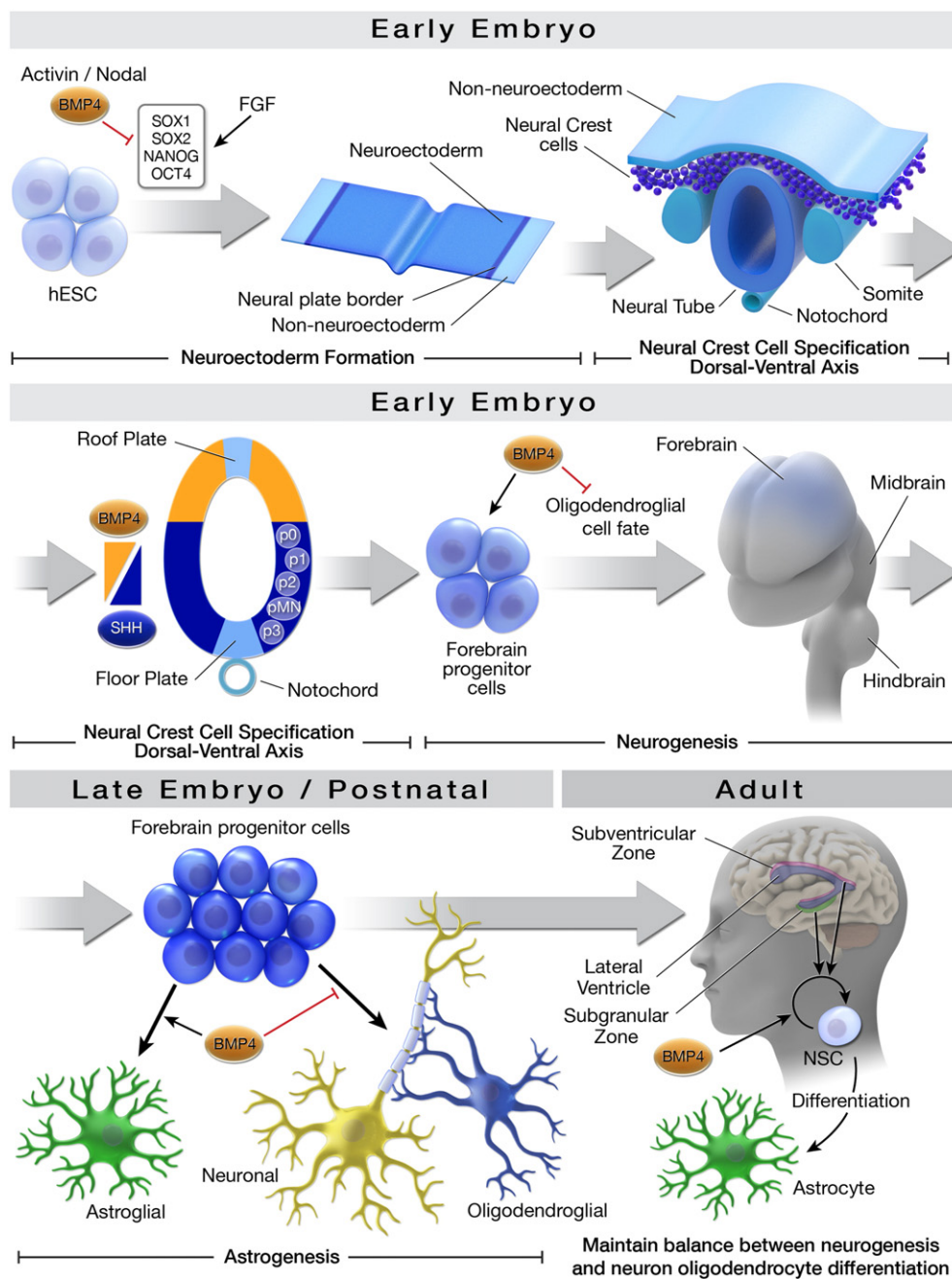
MAPK/ERK activation. Signal crosstalk between BMP4 and WNT/ $\beta$ -catenin, with a WNT/ $\beta$ -catenin signaling activator increasing subsequent downstream BMP4 mRNA transcription, is also involved in this process. Increased BMP4 also promotes the Ras-mediated ERK signaling cascade. This occurs synergistically with epidermal growth factor receptor (EGFR) signaling suppression by BMP4, which simultaneously arrests the mitotic effect of EGF [83] on NSCs and allows ERK to activate the TUJ1 promoter to induce neuronal differentiation [84].

### Human Gliomas and BMP4

Human gliomas are among the most aggressive malignancies. These malignancies may arise from GCSCs. BMP4 has been identified as a prognostic marker for human gliomas, and as a potential target for therapeutic treatment. Treatment with BMP4 may drive tumor cells towards a differentiated state, mimicking the role of this molecule during development, and increase tumor chemotherapeutic sensitivity.

### BMP4 Expression and Clinical Significance in Human Glioma

BMP4 is associated with human glioma WHO grade [85], with both BMP4 protein and its signaling components considered novel biomarkers for prognosis and prediction of survival. BMP4 expression is markedly lower in tumor tissue, compared to adjacent normal tissue [86]. It is down-regulated in high grade gliomas, compared to low grade gliomas [85,86]. Moreover, lower levels of BMP4 are associated with a high proliferation index and poor survival [86], while high BMP4 expression is

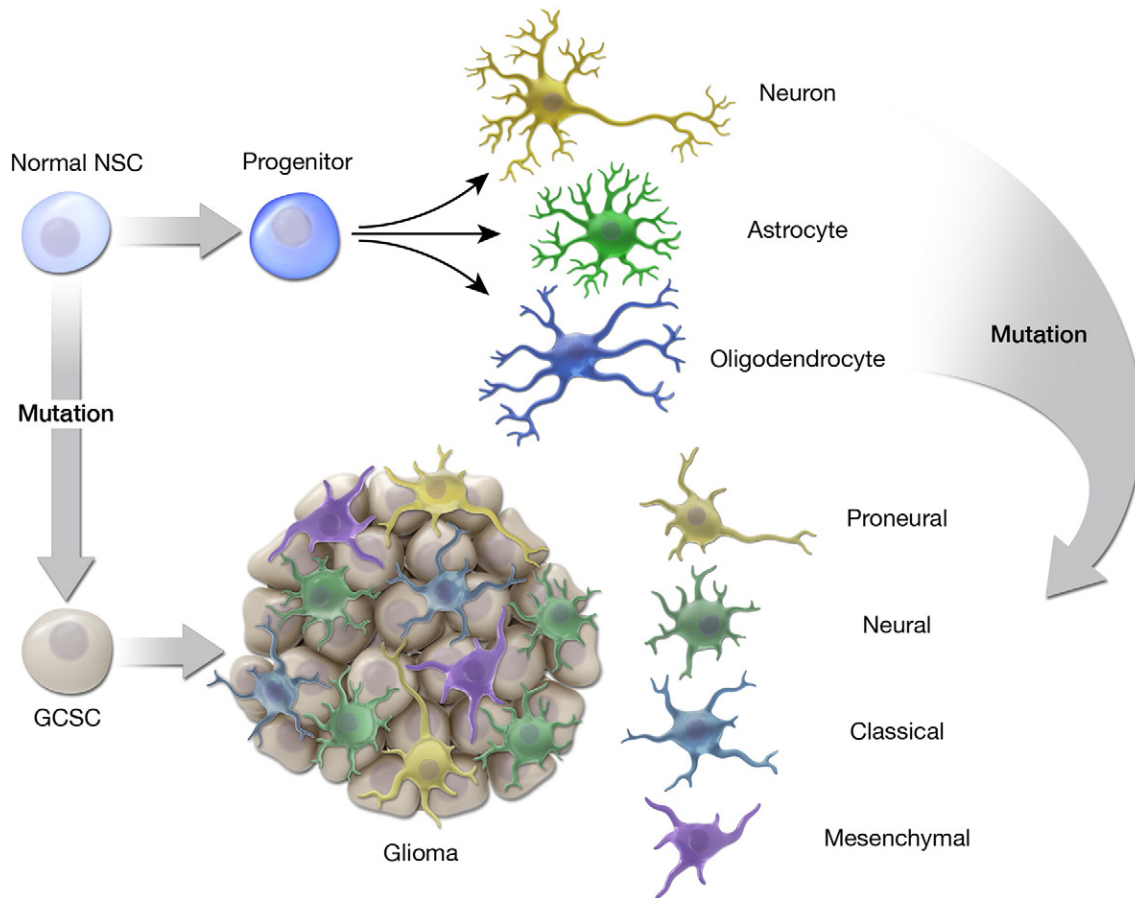


**Figure 2.** BMP4 in CNS development. BMP4 has changing roles during CNS development and is a factor in functional maintenance. BMP4, Nodal and Activin inhibit reprogramming factors Sox2, Nanog and Oct4 in human embryonic stem cells to induce neural plate formation. The neural plate folds to create the neural groove, which pinches off from the overlying ectoderm to form the neural tube. In this stage, the ectoderm and neural tube roof plate (RP) continue to secrete BMP ligands, which directly oppose Shh secreted ventrally from the notochord and floor plate (FP). BMP4 and Shh gradients establish the dorsal-ventral axis, with the area of intermediate signaling specifying NCCs, which will form the peripheral, sympathetic, and sensory nervous systems. Following neural induction BMP4 secreted from the ectoderm and neural tube roof plate is involved in neural patterning of CNS regions, including forebrain, cerebellum, and dorsal spinal cord. During forebrain development, BMP4 promotes neuronal lineage commitment while inhibiting oligodendrocyte formation. In late embryogenesis and postnatally, BMP4 signaling changes to promote astroglial commitment while repressing commitment to neuronal and oligodendroglial lineages. In adulthood, tight regulation of BMP4 signaling is critical for maintenance and differentiation of NSC populations in the sub-ventricular zone (SVZ) and sub-granular zone (SGZ).

associated with lower mortality [85]. Molecular background may be critical for function, as BMP4 expressed in gliomas with a mutation in the isocitrate dehydrogenase 1 (IDH1) gene [87], or in gliomas with a proneural or G1 subtype, indicates better prognosis [5,88,89].

Examining components of the BMP4 signaling pathway suggests a beneficial outcome for glioblastoma patients when this pathway is active. Inactivation of BMP4 signaling including decreased R-SMAD

phosphorylation is associated with high grade gliomas, such as anaplastic astrocytoma and glioblastoma, compared to normal brain and low grade astrocytoma [90]. BMPRIIB receptor expression is down-regulated in high grade gliomas. Patients with a low R-SMADs/total Smad1/5/8 ratio have shorter survival, highlighting P-Smad/BMPRIIB reversely correlates with glioma grade [90]. In addition, epigenetic silencing of BMPRIIB impairs GCSCs astroglial



**Figure 3.** Brain tumors derived from NSCs. NSCs are able to self-renew and to give rise to multipotent committed progenitors and mature differentiated cells that form organized brain parenchyma. Tumor-initiating mutations may occur in NSC, driving them to be glioma cancer stem-like cells (GCSCs), which can give rise to distinct lineages of gliomas. Tumor-initiating mutations may also take place in differentiated cells resulting in generation of committed gliomas.

differentiation due to BMP4 signaling loss, reinforcing that this loss contributes to human glioma tumorigenesis [21].

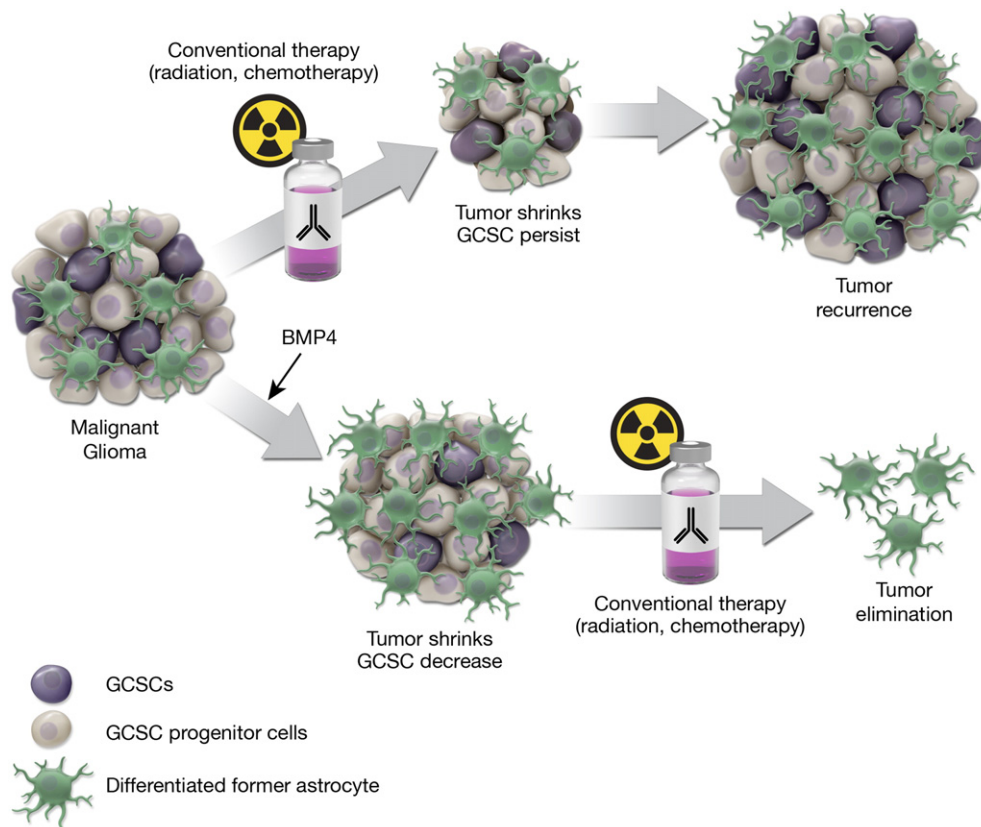
### *BMP4 and Human Glioma Tumorigenesis*

GCSCs are believed to initiate human glioma tumorigenesis [7,91–93] as shown in Figure 3. Cancer stem cells expressing the stem cell marker CD133 have been isolated from astrocytic brain tumors [94], these cells are capable of self-renewal in vitro and of propagating the original tumor in vivo [91,95–97]. GCSCs appear to arise by transformation of proliferating NSCs [98], as these cell types share common signal pathways [96,99] and are phenotypically similar. However, compared to NSCs, GCSCs exhibit dysfunctional patterns with enhanced self-renewal and compromised differentiation [91,93,100,101]. Moreover, multiple signal pathways including BMP signaling, required to enhance stemness and improve cell survival, and consequently enable tumorigenesis [11,91], were up-regulated in GCSCs.

BMPs regulate proliferation, differentiation, and apoptosis in NSCs as aforementioned. Levels and timing of expression are critical for proper development. For example, BMP4 promotes proliferation of NSCs cultured from the anterior SVZ at low concentration, but inhibits proliferation at high concentration. BMP4 enhanced neuron commitment of these cells at an early stage but inhibited it at a later stage [102]. Interactions between BMP and other signaling pathways including Wnt/ $\beta$ -catenin, basic helix–loop–helix (bHLH) and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) are essential for appropriate developmental

outcomes [103,104]. For example, BMP expression, induced by Wnt signaling, drives NSCs towards an astroglial lineage [105].

Similar to induction of an astroglial fate in NSCs, BMPs also drive astroglial differentiation in GCSCs, and thus may inhibit GCSC tumorigenesis. Exogenous BMP4 decreases GBM cell proliferation and drives astroglial differentiation in CD133 positive GCSCs [22,106,107]. Suppression of EGFR, which is amplified in a large proportion of primary GBMs, may be involved in this process [108]. In vivo delivery of BMP-4 inhibits brain tumor growth with a resultant decrease in mortality [22]. Primary human GCSCs expressing EZH2 show enhanced tumorigenicity, possibly due to epigenetic silencing of the BMP type I receptor, resulting in BMP-induced differentiation bypass [109]. BMPRI and R-SMAD expression levels inversely correlate with tumorigenesis in human high grade gliomas [90]. In the presence of Gremlin 1, a BMP antagonist, GCSC differentiation is inhibited, while self-renewal and tumorigenic potential are maintained [110]. BMP4 may also act on proliferation via other mechanisms such as down-regulation of cyclin D1 and induction of apoptosis through inhibition of Bcl-2 and Bcl-XL and induction of Bax [111]. Altogether, these findings indicate that BMP4 is critical in regulating glioma tumorigenesis. They also highlight the need to identify molecular mechanisms by which BMP4 signaling controls GCSC tumorigenesis. Identifying these mechanisms will define targets to enhance therapeutic efficacy and improve patient prognosis.



**Figure 4.** BMP4 improves therapeutic efficacy for malignant gliomas. GCSCs exhibit chemo- and radio-resistance in response to conventional therapy, and thus the patient manifests tumor recurrence. BMP4 targets GCSCs and drives them to differentiate into cells with increased sensitivity to conventional therapy. This markedly improves therapeutic efficacy, and hence patient prognosis.

#### *BMP4 as a Therapeutic Target in Human Glioma*

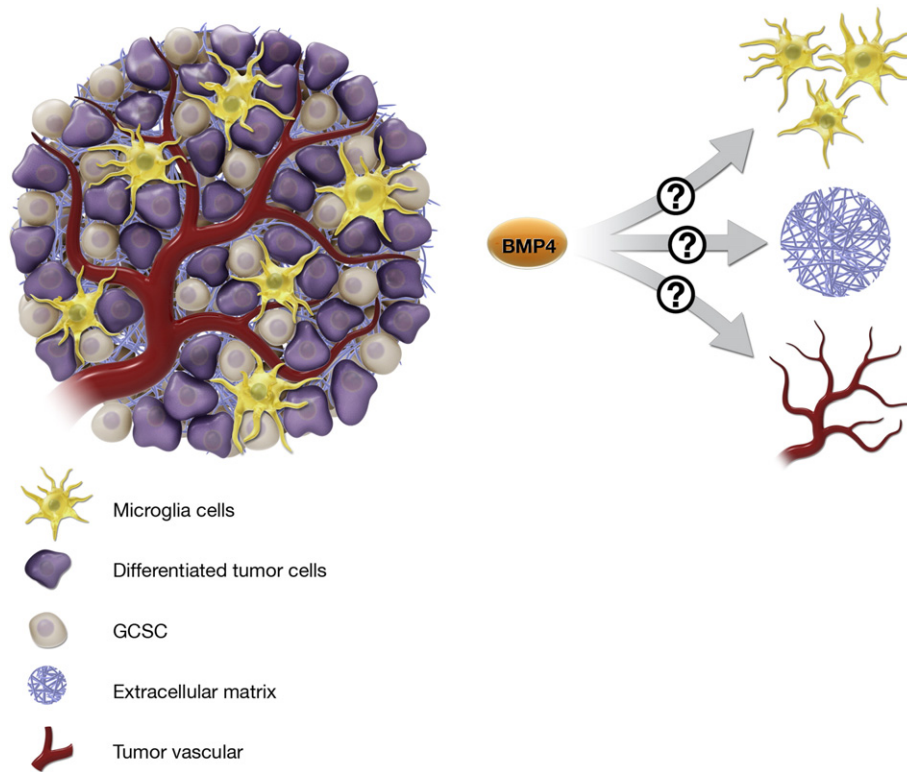
High grade gliomas contain GCSCs [91,93,97], which present a major obstacle for successful chemotherapy and radiation therapy [3,8,112]. GCSCs contribute to glioma recurrence and therapeutic resistance through multiple mechanisms including induction of the multidrug resistance (MDR) phenotype, alteration of DNA damage response mechanisms, and the Notch signaling pathway [8,113,114]. GCSCs express MDR through reduction of drug uptake or by effluxing cytotoxic drugs through up-regulation of ATP-binding cassette (ABC) transporters [115]. One of these ABC transporters, ABCG2, is regulated by the PTEN/PI3K/Akt pathway. Gliomas with PTEN loss have increased GCSC populations [114]. GCSCs also exhibit abnormal cell death pathways including overexpression of anti-apoptotic proteins or down-regulation of pro-apoptotic factors [116].

BMP signaling drives NSC cell-cycle exit and promotes NSC astroglial differentiation [20]. Similarly, BMP4 activates GBM-derived stem cell astrocyte differentiation both in vitro and in vivo [22], indicating BMP4 and its signaling pathway could be promising targets for differentiation therapy. BMP4 reverses the GBM cell MDR phenotype to sensitize glioblastoma response to temozolomide [117], a first-line chemotherapeutic treatment against glioblastoma. BMP4 in combination with bevacizumab, a humanized monoclonal antiangiogenic antibody against vascular endothelial growth factor A (VEGF-A), reduces glioma tumor size and invasion [118]. Activating BMP signaling differentiates GCSCs in experimental models of human GBM and increases chemosensitivity [22]. BMP4 markedly reduces the formation of spheres by CD133+ GCSCs in vitro and human GBM-bearing mice treated with BMP4 induce CD133+ GCSC differentiation [55]. In addition, BMP4-treated GBM xenografts

produce smaller tumors and substantially prolonged survival, compared to untreated groups [22]. Taken together, BMP4 may act as a key inhibitory regulator of glioma tumorigenesis to improve therapeutic efficacy in combination with conventional chemotherapy and radiation therapy.

Unfortunately, the efficacy of BMP4 and chemotherapeutic reagents is limited due to the presence of the blood–brain barrier (BBB) which controls the diffusion of endogenous molecules and xenobiotics. Recent studies show favorable results using BMP4 bioengineered with human NSC virus and other materials for tumor-targeted drug delivery [119–122]. For example, oncolytic vaccinia virus overexpressing BMP4 promotes cell differentiation of GCSCs obtained from GBM biopsies, and improves survival [122]. Moreover, systemically delivered BMP4 overexpressing human adipose-derived mesenchymal stem cells (hAMSCs) decreased migration and proliferation, and induced differentiation of GCSCs in vitro and improved median survival [121,123]. In addition, bioengineered polymeric nanoparticles (NPs), hAMSCs and BMP4 (BMP4/NP-hAMSCs) administrated intranasally pass through the BBB, target brain tumors, and secrete BMP4 to improve survival of GBM derived from GCSCs [120]. These studies demonstrate that novel therapeutic application of BMP4 can target GCSCs to increase therapeutic efficacy and improve treatment outcome.

In summary, BMP4 decreases GCSCs, by promoting GCSC differentiation, which in turn increases human glioma response to chemotherapy and radiation therapy (Figure 4). Thus, BMP4 or bioengineered BMP4 stem cell targeting therapy, in combination with conventional surgery, chemotherapy and radiation therapy represents a novel strategy to increase glioma therapeutic efficacy, decrease glioma recurrence and improve patient survival. Further



**Figure 5.** The microenvironment is critical in tumorigenesis and therapeutic response of malignant gliomas. BMP4 effects on GCSCs must be fully investigated both independently and in the context of the microenvironment. Aspects of the tumor microenvironment which may be influenced by BMP4 and in turn impact cancer cell proliferation and progression include the extracellular matrix, microglia, which act as brain tumor immune response cells, and tumor microvasculature.

investigation should also examine GCSC heterogeneity and responsiveness to BMP4.

### Prospective and Future Directions

Progress in the treatment of human malignant glioma has been practically non-existent in the past decades. Failure to eradicate GCSC resistance to both chemotherapy and radiation therapy is a major factor for tumor recurrence and poor prognosis. BMP4 targets and promotes GCSC differentiation and inhibits proliferation. Elucidating cellular mechanisms associated with BMP4 regulation of genetic and epigenetic signals associated with GCSC proliferation and differentiation, in combination with determining mechanisms of GCSC chemotherapy and radiation therapy may lead to innovative interventions. These interventions should be integrated into a comprehensive treatment approach, as human malignant gliomas are highly vascularized, aggressive, and diffusely infiltrating, features which also play a role in therapeutic failure [124]. BMP signals modulate both cancer cells and the tumor microenvironment, BMPs can promote or suppress cancer proliferation and progression depending on cellular contexts. Future therapeutic efforts must take into account and target extracellular matrix, microglial immune cells and additional cells that contribute to the microenvironment which allows for GCSC maintenance (Figure 5).

### Conflicts of Interests

The authors declare no conflict of interest.

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