BMP SIGNALING IN HIGH GRADE GLIOMAS

By

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DEDICATION

For Katy Dhimitri and Barb Hover, two of the most courageous, strongest women I have ever known. The memories of these women and their fights against cancer are and will continue to be the driving force behind my research.

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ABBREVIATIONS

ACVR1	Activin A receptor type I
ACVR1b	Activin A type receptor IB
ACVRII	Activin A receptor type IIA
ACVRIIB	Activin A receptor type IIB
ACVRL1	Activin A Receptor Type IL
AMH	Anti-Mullerian hormone
AMPK	AMP-activated protein kinase
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
BMPR1a	Bone morphogenetic protein receptor type IA
BMPR1b	Bone morphogenetic protein receptor type IB
BMPR2	Bone morphogenetic protein receptor II
BTSC	Brain tumor stem cell
CCGA	Chinese Glioma Genome Atlas
CDK	Cyclin-dependent kinases
CNS	Central nervous system
DCX	Doublecortin
DIPG	Diffuse Intrinsic Pontine Glioma
DLL3	Delta-Like 3
DMH1	Dorsomorphin homologue 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
FACS	Fluorescence activated cell sorting
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Grem1	Gremlin 1
GSC	Glioma stem cell
HGG	High-grade glioma
Id1-4	Inhibitor of DNA binding 1-4
IDH1	Isocitrate dehydrogenase

IHC	Immunohistochemistry
Itgb4,7	integrin beta subunits 4 and 7
JPS	Juvenile Polyposis Syndrome
KO	Knockout
Kras	Kirsten rat sarcoma viral oncogene
MERTK	Mer Tyrosine Kinase proto-oncogene
MES	Mesenchymal
miR-656	Micro-RNA 656
MMC	Mitomycin-C
mTom	mTomato
NCI	National Cancer Institute
NeuroD	Neuronal differentiation 1
NF1	Neurofibromin 1
NKX2.2	NK2 Homeobox 2
NRTN	Neurturin
NSC	Neural stem cell
NSCLC	Non-small cell lung cancer cells
OAZ	O/E-associated zinc finger protein
Olig1	Oligodendrocyte lineage transcription factor 1
Olig2	Oligodendrocyte lineage transcription factor 2
p53	Tumor protein p53
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphoinositide 3-kinase
PN	Proneural
Prom-1	Prominin 1
p-Smad	Phosphorylation of Smad
PTEN	Phosphatase and tensin homolog
RB1	Retinoblastoma gene
REMBRANDT	REpository for Molecular BRAin Neoplasia DaTa
REST/NRSF	RE1 silencer of transcription/neuron-restrictive silencer factor
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SEM	Standard error of the mean
SGZ	Subgranular zone

SIP1	Smad Interacting Protein 1
Smad	Mothers against decapentaplegic homolog
Smurf1,2	Smad ubiquitination regulatory factors 1 and 2
SnoN	Ski-related novel protein N
SOX2	SRY (sex determining region Y)-box 2
SVZ	Subventricular zone
TCGA	The Cancer Genome Atlas
TGFβ	Transforming growth factor beta
TIC	Tumor initiating cells
TMA	Tissue microarray
TMZ	Temozolomide
Tob	Transducer Of ERBB2, 1
VEGF	Vascular endothelial growth factor
WHO	The World Health Organization
YKL40/CHI3L1	Chitinase-3-like protein 1 (CHI3L1)

CHAPTER I: INTRODUCTION

INTRODUCTION TO GLIOMAS

Primary brain tumors are responsible for 1.4% of all cancers and 2.4% of all cancer deaths within the United States[1]. Malignant gliomas are some of the most deadly and prominent types of brain tumors. Gliomas are defined as tumors that morphologically resemble glial cells, which include astrocytes, oligodendrocytes or ependymal cells. Glial cells are the most abundant cells within the CNS and serve to maintain, regulate, propagate and support neural cells[2]. Astrocytes support and regulate the environment for neuronal signaling. Oligodendrocytes wrap myelin around axons to promote nerve conduction and ependymal cells are epithelial cells which line the ventricular system[2]. Consequently, gliomas are diagnosed as astrocytomas, oligodendrogliomas, ependymomas, or mixed gliomas[2]. This system was originally developed by Bailey and Cushing in 1926 in which tumors were classified based on the appearance of the predominant cell type and its similarity to developmental counterparts[3]. Within the central nervous system (CNS), gliomas account for approximately 28% of all tumors and 80% of all malignant CNS tumors[4].

The World Health Organization (WHO) has created a system to classify gliomas into four grades based on histological features that correlate with patient outcomes[5]. Gliomas are graded based on the histological features of the tumor including hypercellularity, vascularity, necrosis, hemorrhaging, nuclear atypia and the presence of mitotic figures[6]. Grade I and II gliomas are considered low grade gliomas, as they are relatively slow growing tumors and show low proliferative activity. Grade III and IV gliomas are considered high grade gliomas (HGG) and are rapidly growing, highly infiltrative tumors[6]. Grade III gliomas are distinct from low grade gliomas by the presence of increased cellularity, mitotic activity and nuclear atypia. In addition to those characteristics, grade IV gliomas show microvascular proliferation and necrosis [6].

Astrocytomas account for approximately 75% of all gliomas[4], the most common being a grade IV glioma, known as glioblastoma multiforme (GBM). GBMs are devastating tumors with a median survival of 15 months and only 5% of patients surviving 5 years past diagnosis[3]. GBMs account for approximately 46% of all malignant CNS tumors with 10,200 cases predicted in 2015 alone[4]. GBMs are divided into primary tumors and secondary tumors. Approximately 90% of GBMs are primary tumors, which develop de novo, most commonly in patients 50 years or older. Secondary GBMs begin as low grade gliomas and develop into GBMs over a period of years[1].

Diagnosis & Treatments

Symptoms of malignant gliomas vary based on location of the tumor, but can include headaches, seizures, nausea, changes in speech, vision, hearing or balance, memory loss or personality changes[1]. The current standard of treatment for malignant gliomas includes surgical resection, radiotherapy and chemotherapy[6]. As mentioned previously, HGG are known to be highly invasive tumors, therefore surgery generally cannot completely eliminate the tumor, and approximately 90% of GBM patients show recurrence at the original tumor site[6,7]. Both radiotherapy and chemotherapy have considerably increased the mean survival compared to surgery alone in GBM patients. Radiotherapy alone increased survival time from 3-4 months to 7-12 months[6]. Radiotherapy in combination with Temozolomide (TMZ), a DNA alkylating agent, increased the median survival to 14-15 months[6,8]. Another chemotherapeutic option is the implantation of biodegradable wafers releasing carmustine, an alkylating agent, into the brain following surgery to kill remaining tumor cells. This approach has also been shown to increase survival from approximately 12 months to 14 months[6]. However, despite all treatments, the vast majority of GBMs recur and the median time to tumor progression after treatment is 6.9 months[6]. Even with aggressive therapy, the average survival time remains at 14-15 months and the 10 year survival rate is only 2.6%. Clearly a better understanding of the underlying tumor biology is needed to develop more effective treatments.

Commonly Dysregulated Signaling in Gliomas

GBMs are extremely heterogeneous at both the histological and molecular level, intra and intertumorally[1,9–11] impeding the development of effective therapies. However, there are several pathways that are known to be dysregulated in the majority of GBMs. One of the most common genetic changes is the dysregulation of growth factor signaling through amplification, copy number gain or mutation of receptor tyrosine kinases (RTK), most commonly being the epidermal growth factor receptor (*EGFR*) and platelet-derived growth factor receptors (*PDGFR*)[1,12] These genetic changes lead to upregulated RAS and mitogen-activated protein kinase (MAPK) signaling, effectively increasing proliferation[1]. EGFR amplification is seen in 40-50% of primary GBMs[1] and PDGFR-α,β amplification is seen in approximately 20-30% of primary GBMs[1]. Frequently both the growth factor receptors and ligands are found to be increased within malignant gliomas, commonly combined with a mutation resulting in constitutive activation in EGFR [1,13]. In addition, the tumor suppressing genes, tumor protein p53 (*TP53 or p53*) and retinoblastoma gene (*RB1*) are frequently inactivated[1]. p53 signaling is estimated to be altered in 87% of GBMs and RB signaling is altered in approximately 78% of GBMs[12]. Other common alterations include phosphoinositide 3-kinase (PI3K) mutations and phosphatase and tensin homolog (PTEN) mutations, both critical to cell cycle regulation. Most GBMs contain a combination of alterations and mutations within growth factors, p53 and RB resulting in aggressive tumors that are highly proliferative and invasive[14] [**Figure 1**]. Because the vast majority of GBMs harbor mutations and alterations in p53, RB and RTKs it has been hypothesized that alterations in these pathways are core requirements for the development of GBMs[15].

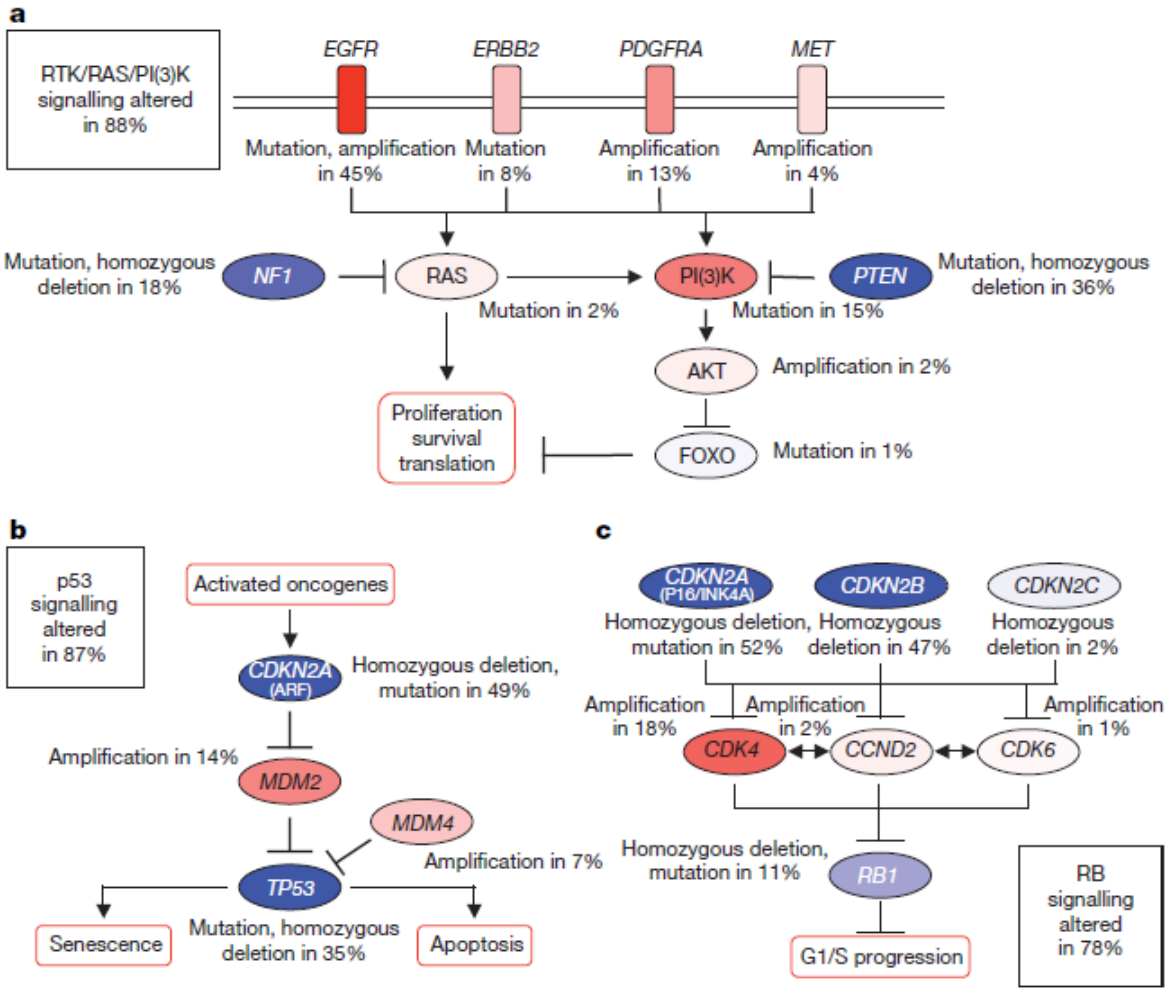


Figure 1. Frequent genetic alterations in three critical signaling pathways

a–c, Primary sequence alterations and significant copy number changes for components of the RTK/RAS/PI(3)K (a), p53 (b) and RB (c) signaling pathways are shown. Red indicates activating genetic alterations, with frequently altered genes showing deeper shades of red. Conversely, blue indicates inactivating alterations, with darker shades corresponding to a higher percentage of alteration. For each altered component of a particular pathway, the nature of the alteration and the percentage of tumors affected are indicated. Boxes contain the final percentages of glioblastomas with alterations in at least one known component gene

GBM Subtypes

Recently with the rapid advancement of sequencing technology and accumulation of genomic data, studies suggest that while GBMs share certain genetic and epigenetic alterations, tumors segregate into subclasses based on gene expression signatures. Two major studies have established subclasses based on extensive genomic profiling, the first published by Aldape's group in 2006, and the second published by the TCGA research network in 2010. Although each study organized subgroups slightly differently based on methodology, two primary subclasses were discovered that display distinct and exclusive markers and phenotypically resemble different stages of neural development. The two principal subclasses are termed Proneural (PN) and Mesenchymal (MES) reflecting the dominant feature of the gene expression pattern within each group. PN tumors most highly express genes associated with neuroblasts, immature neurons and oligodendrocytes including Sox family genes, *DLL3*, *NeuN* and *DCX*[15,16]*NKX2.2*, and *Olig2*. PN tumors also contain the highest percentage of tumors with PDGFRα alterations including overexpression and point mutations, IDH1 point mutations and loss of or mutations in *p53*. PN tumors are associated with younger patients, grade III astrocytomas and secondary GBMs[15,16]. Phillips *et al* showed an association between PN tumors and longer survival, however PN tumors did not show any survival benefit from therapy[16]. MES tumors are associated with undifferentiated neural stem cells[17,18]. MES tumors most commonly display decreased expression or deletion of *NF1* and express mesenchymal tissue and astrocytic markers including *YKL40*, *CD44* and *MERTK*[15]. Furthermore, MES tumors are considered to be more aggressive tumors and show the presence of necrosis and markers of angiogenesis[16]. Phillips *et al* determined that upon recurrence, tumors that shift subtype most typically change from the PN to the MES subtype[16]. Other GBM genomic profiling studies have validated the PN and MES subgroups[19,20]. The gene expression profiles defining these subgroups emphasize the importance of understanding neural stem cells, neural development and the pathways regulating these processes.

Glioma stem cells

It has been a central question in the field of cancer research as to which cells are capable of initiating, propagating and maintaining tumor growth. Recent evidence has shown that within many types of cancer, there is a small population of cells, termed cancer stem cells, with properties similar to normal stem cells and upon transplantation are capable of forming phenotypically similar tumors[21,22]. In the 1960s and 1970s key discoveries were made which transformed the field of cancer biology and cancer stem cells. Evidence for the clonal nature of cancer cells was first discovered in leukemia in the 1960s when investigators found a chromosomal abnormality that was present in almost all dividing leukemia cells, showing derivation from a single cell of origin[22]. During this time many other investigators made the observation that cancer cells isolated from hematological malignancies and solid-organ tumors varied in their ability to proliferate, self-renew and initiate tumor growth[22,23]. Following these discoveries, populations of stem-like cells have been isolated from many types of cancer including breast, melanoma, colon and prostate cancer. In the early 2000s, work from Peter Dirks, Harley Kornblum and Dennis Steindlers' laboratories published evidence for brain tumor stem cells. These groups were able to isolate a population of cells from glial tumors that were phenotypically similar to normal neural stem cells[24–27]. These cells variously termed glioma stem cells (GSC), brain tumor stem cells (BTSC) or tumor initiating cells (TIC) show stem-like characteristics, are multipotent, able to self-renew, and are regulated by many of the same pathways as normal neural stem cells[28,29]. These stem-like cells are frequently characterized by the expression of markers expressed by normal neural stem cells including CD133, Nestin, SOX2, and Olig2. In 2004 Singh *et al* published a study in which only GSC, identified by the expression of CD133, formed tumors which resembled the original tumor, whereas CD133-negative cells, or the non-stem population, were not able to form tumors upon transplantation in NOD-SCID mice[26]. Remarkably, 100 CD133-postive cells were able to form tumors whereas injections containing 10^5 CD133-negative cells were not. Since then, many other studies have

identified a small population of stem-like cells within gliomas and have shown that GSC are able to recapitulate the original tumor upon orthotopic implantation in murine models[14,30].

However, two major questions remain in the field of glioma stem cells. It is currently unknown if GSC arise from neural progenitor cells or if differentiated cells dedifferentiate and gain stem-like characteristics during the development or progression of the tumor. Secondly, it is unknown if GSC represent a cell of origin for human gliomas [**Figure 2**]. We and others have shown that differentiated cells can regain expression of stem cell markers such as Olig2 and Nestin upon oncogenic transformation and that both GSC and differentiated cells are capable of forming high grade gliomas upon oncogenic stimulation[31,32]. However, more in-depth research needs to be conducted to thoroughly answer these questions. Regardless of their origin, or whether GSC are the true initiating cells in human gliomas, it is generally accepted that populations of stem-like cells are present within HGG and help to propagate the tumor. Depending on methodology the estimate varies, but it has been reported that less than 1-30% of the tumor is comprised of glioma stem cells[1,26,33]. Even though it is a small population, GSC are thought to be resistant to the current therapies contributing to tumor recurrence[14,34]. Therefore it follows that targeting and eliminating this population would significantly increase survival. One approach is to use the knowledge we have gained about the regulation of normal neural stem cells to target the GSC population specifically.

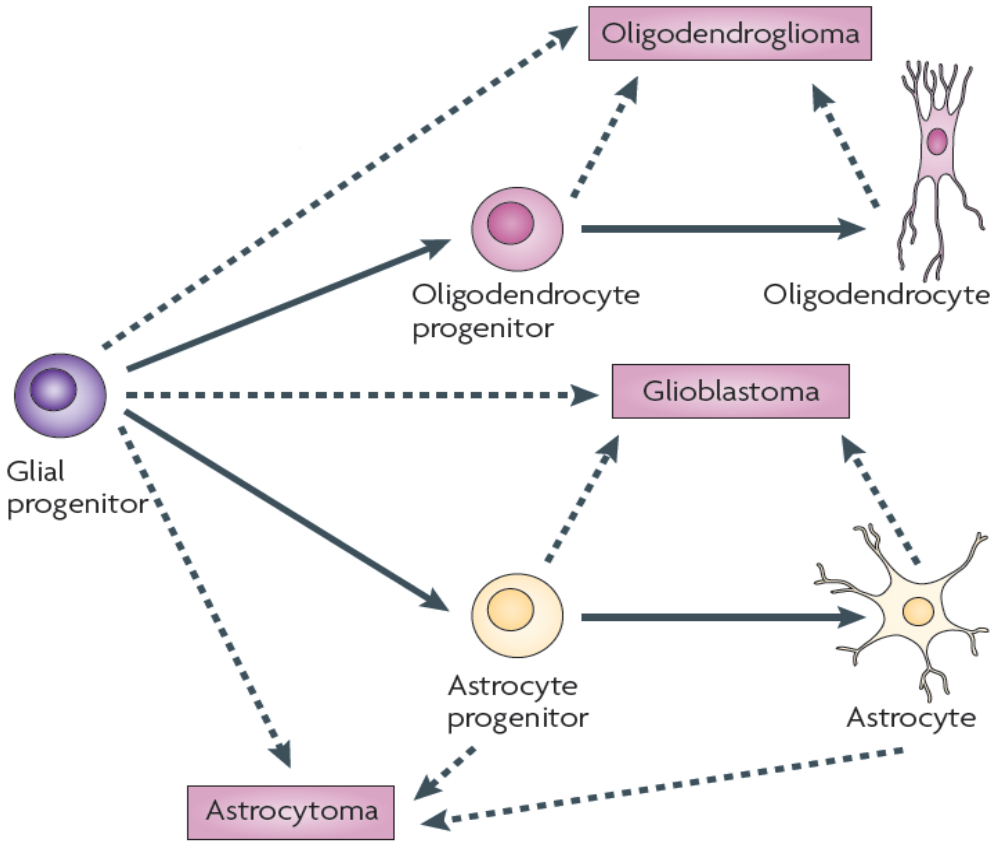


Figure 2. The neuroglial lineage tree

Self-renewing, common progenitors are thought to produce committed neuronal and glial progenitors that eventually differentiate into mature neurons, astrocytes and oligodendrocytes. Although the precise cells of origin for diffuse glioma variants remain largely unknown, a selection of likely candidates for each (dashed arrows) is indicated. Used with permission. Modified from original figure[179]

BMP SIGNALING

Pathways crucial to development are frequently involved in cancer progression or suppression. One such pathway is the bone morphogenetic protein (BMP) pathway. BMPs were discovered by Dr. Marshall Urist in 1965 as unknown molecules with the ability to induce bone formation at ectopic sites; however the specific proteins remained unknown until the 1980s[35,36]. Even though BMPs were originally discovered as bone-inducing factors, BMPs play critical roles in embryogenesis, skeletal formation, hematopoiesis and neurogenesis and are known to affect most cell types by regulating cell growth, differentiation, cell-fate determination, proliferation, apoptosis, and movement. Furthermore, BMPs are crucial to the development of almost all organs and tissues including the nervous system, lungs, kidney, skin and the basic embryonic body plan[37,38].

BMP signaling molecules are members of the transforming growth factor beta (TGFβ) family which also includes activins, inhibins, nodal, myostatin and anti-Mullerian hormone (AMH)[39]. The BMP family is comprised of over 30 factors including ligands, type I and type II serine/threonine kinase receptors, and Smads and is the largest subgroup within the TGFβ family[40]. BMP ligands are divided into 4 groups based on their sequence similarity: BMP2/4, BMP5/6/7/8a/8b, BMP9/10 and BMP12/13/14. All BMP ligands begin as large precursor proteins which are cleaved to form active dimers[38]. These active ligand dimers then bind to type I and type II receptors. There are 4 primary BMP type I receptors, bone morphogenetic protein receptor IA (BMPR1A), bone morphogenetic protein receptor IB (BMPR1B), activin A receptor type I (ACVR1) and activin receptor-like kinase 1 (ACVRL1) and 3 type II receptors, bone morphogenetic protein receptor type II (BMPR2), activin A receptor type IIA (ACVR2) and activin A receptor type IIB (ACVR2B). Type I and type II receptors are similar in structure, containing a single transmembrane domain, a short extracellular domain and a single intracellular serine-threonine kinase domain[39]. A phenomenon termed ligand-receptor

promiscuity is particularly evident within the BMP family in which receptors are capable of binding to multiple ligands and likewise, ligands bind to multiple type I and type II receptors[41]. As a result of this promiscuity, BMP ligands signal through heterotetrameric complexes of type I and type II receptors. In the canonical response to ligand binding, the type II receptor kinase phosphorylates the type I receptor which then phosphorylates the BMP regulatory Smads, Smad1/5/8 [42]. The type I receptor phosphorylates the regulatory Smads through direct contact. The MH2 domain of the regulatory Smads1/5/8 is sequence specific for BMP type I receptors and binds directly to the receptor [39]. Upon phosphorylation, the Smads are released from the receptor and bind to the co-Smad, Smad4[43]. The Smad complex is then translocated to the nucleus for transcriptional regulation, binding directly to specific DNA sequences containing the Smad-binding element (SBE), DNA-binding proteins or DNA-binding cofactors to activate transcription factors, co-activators and repressors[42,44]. The inhibitor of differentiation genes, *Id1-4*, are induced by BMP signaling in most cells and are some of the most important targets of BMP signaling [42]. Other common early response genes include *OASIS* and *Snail*[39][**Figure 3**].

Regulation of BMP Signaling

The BMP pathway is tightly regulated both intracellularly and extracellularly by inhibitory Smads, ligand antagonists, signaling crosstalk, and other inhibitory molecules. The inhibitory Smads, Smads 6 and 7 are induced in response to active BMP signaling creating a negative feedback loop. Smad6 is the primary BMP inhibitory Smad and is known to be upregulated within 2 hours of BMP signaling[39]. The inhibitory Smads bind to type I receptors, inhibiting the regulatory Smads from being phosphorylated and activated. In addition, Smad6 binds to regulatory Smads and inhibits Smad4 binding, effectively inhibiting translocation to the nucleus[39,43]. There are over 20 BMP antagonists which bind to ligands extracellularly and inhibit the ligand from binding to BMP receptors [45–48]. Each antagonists can bind to multiple types of ligands[49]. One of the most important BMP antagonists in the brain is Noggin which is necessary

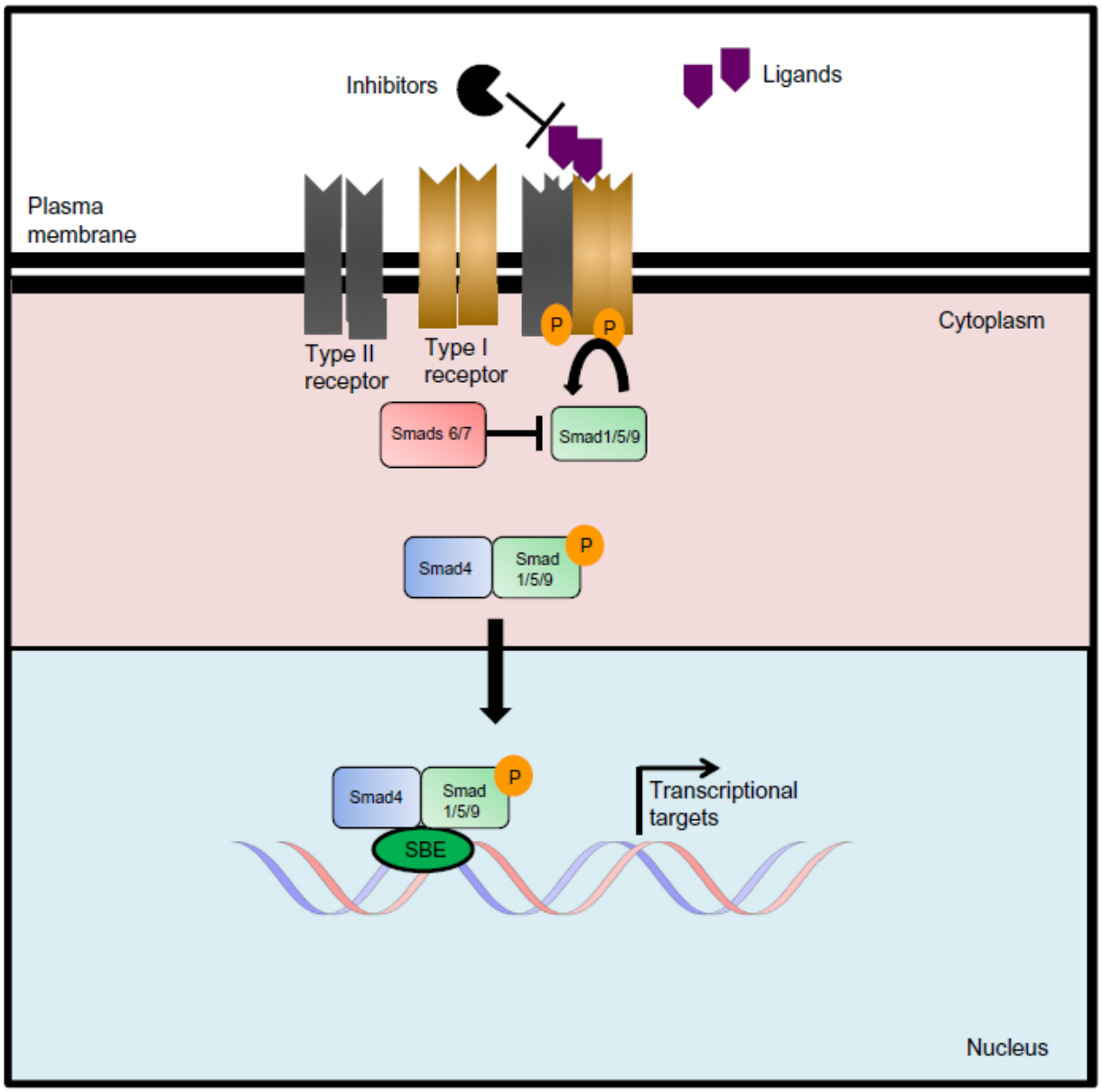


Figure 3. Canonical BMP signaling

Figure 3. Canonical BMP Signaling

The TGFβ family consists of cytokines that signal through serine/threonine kinase receptors. Activated ligands bind to type I and type II serine-threonine kinase receptor complexes, which transduce the signal downstream through the phosphorylation of pathway-specific regulatory Smad proteins (SMADs-1/5/9 are associated with BMP signaling and Smads 2/3 are associated with TGFβ signaling). Generally, the type II receptors phosphorylate the type I receptors, which in turn, phosphorylate the regulatory Smads. Phosphorylated regulatory Smads form a complex with Smad4, the co-Smad, and the complex is translocated to the nucleus for transcriptional regulation. Inhibitory Smads (SMADs 6 and 7) compete with regulatory Smads through both competition for phosphorylation by the type I receptors and binding to Smad4. Signaling antagonists work upstream of the signaling cascade by inhibiting ligands from binding to the receptor complex. Used with permission[50]

for inhibiting BMP signaling during brain development and in maintaining neurogenic niches in the adult brain[45,51].

There are several other inhibitory molecules including BAMBI, Smurfs and nuclear proteins. BMP and activin membrane-bound inhibitor (BAMBI) is a pseudoreceptor that interacts with type I and II receptors, blocking active signaling[39]. The Smad ubiquitination regulatory factors 1 and 2 (Smurf1,2), are ubiquitin ligases which inhibit BMP signaling by targeting Smad1 and 5 for degradation[43]. In addition, there are many co-factors within the cell such as the nuclear proteins Ski, SnoN, Tob, SIP1, and OAZ which all interact with activated regulatory Smads and generally serve to inhibit BMP signaling[43]. One other source of pathway regulation is through crosstalk with other signaling pathways. The TGFβ pathway regulates BMP signaling through multiple mechanisms. The regulatory Smads activated by TGFβ and BMP signaling both require Smad4 for translocation to the nucleus, therefore competition for Smad4 antagonizes TGFβ and BMP signaling [42]. In addition, TGFβ signaling can affect the expression of the inhibitory Smads, either positively or negatively regulating BMP signaling[42]. Additionally, many other developmental pathways including Notch, Wnt and Hedgehog directly interact with and regulate BMP signaling components[42]. As described above, BMP signaling is very complex and tightly regulated by varied receptor-ligand complexes with a host of co-factors and inhibitors resulting in many diverse downstream effects[42,43,52].

BMP and Neural Development

Bone morphogenetic proteins, as described in the nomenclature, are morphogens, which are factors that diffuse away from their source creating a concentration gradient. BMPs not only regulate processes in a concentration or dose dependent manner[38], but also elicit different effects depending on developmental stage, cell type and the surrounding microenvironment. As mentioned previously, BMPs are essential for the development of almost all organs and tissues[37,38]. Homozygous knockouts of BMP2, 4, 8b, BMPR1A, and BMPR2 are

all embryonic lethal, evidence that BMP signaling is critical to development[39,49,53]. Many reviews have detailed the many roles of BMP signaling in full body development[38,53–55], however here we focus on BMP signaling on neural cells during development.

BMPs and their function are highly conserved among nematodes, arthropods and vertebrates, and many informative studies have been completed in *Drosophila melanogaster* and *Xenopus*[43]. Using large scale mutant screens in *D. melanogaster* and zebrafish, the BMP pathway was discovered to regulate dorsal-ventral patterning[56]. As further characterized in *Xenopus*, gradients of BMP and BMP-antagonists are necessary for dorsal-ventral patterning and the formation of neural tissue[51,55]. As later shown in mice, BMPs and BMP antagonists are necessary for neural tube and forebrain development[51]. The extensive role of BMP signaling in the CNS during development has been detailed in several reviews[47,55,57].

BMP and Neural Stem cells

BMP signaling plays a critical role on neural stem cells at all stages of development and adulthood. As shown in rodent models, neural stem cells respond in different manners to BMP signaling depending on receptor availability and stage of brain development[58]. BMP signaling instructs neural stem cell proliferation, apoptosis, and cell specification in a temporal and concentration dependent manner[51,58]. The BMP receptors, BMPR1A, BMPR1B and BMPR2 are expressed on neural stem cells at different times during CNS development and even though BMPR1A and BMPR1B are structurally similar, these receptors regulate neural cells differently and cannot compensate for one another[47].

In the simplest terms, neural stem cells go through three stages during development, the expansion stage and the neurogenic phase followed by the gliogenic phase modulated by BMP signaling at each stage[59]. In the first stages of development, BMP signals through the BMPR1A receptor of neural stem cells to induce proliferation and dorsal identity. Next, during early gestation, BMPR1B is expressed on neural stem cells and induces mitotic arrest through

expression of the CDK inhibitor p21 and apoptosis. After approximately E11.5, the primary response to BMP signaling is neuronal differentiation[59]. Around E16 and continuing in the postnatal brain, BMP signaling promotes glial differentiation[58,59].

In the postembryonic brain, BMPR1A is expressed at higher levels and more broadly than BMPR1B or BMPR2. In the human adult brain, normal neural stem cells are found within two defined regions of neurogenesis, the subgranular zone (SGZ) and the subventricular zone (SVZ), the SVZ being the largest of the germinal regions[60]. Within the SVZ, strict regulation of BMPs and Noggin creates the neurogenic niche. Noggin is expressed by the ependymal cells lining the ventricle and the BMPs 2,4,7, BMPR1A, BMPR1B and BMPR2 are expressed by cells within the SVZ[47,51] [**Figure 4**]. In the SVZ, BMP signaling promotes astrocytic differentiation, inhibits the generation of neurons or oligodendrocytes while decreasing proliferation of cells[47,51]. Although less understood, it is known that a balance of BMPs and Noggin is required to maintain the neurogenic niche in the SGZ as well[47].

It should be noted that BMP signaling consistently downregulates oligodendrocyte differentiation during development and throughout adulthood[58]. BMP signaling induces the expression of the ID family of genes which negatively regulates basic helix-loop-helix transcription factors. BMP induction of ID2 and 4 is known to sequester Olig1 and Olig2, in effect inhibiting oligodendrocyte differentiation[47,61]. Similarly, transcription factors known to promote neurogenesis such as Mash1, neurogenin and NeuroD are negatively regulated by the ID genes[47]. Furthermore, BMPs induce the expression of RE1 silencer of transcription/neuron-restrictive silencer factor (REST/NRSF) to suppress neuronal differentiation[62]. As evident here, BMPs can induce a wide variety of effects on NSC through development and into adulthood due to unique and precise combinations of ligands, receptors, inhibitors and regulation of transcriptional factors.

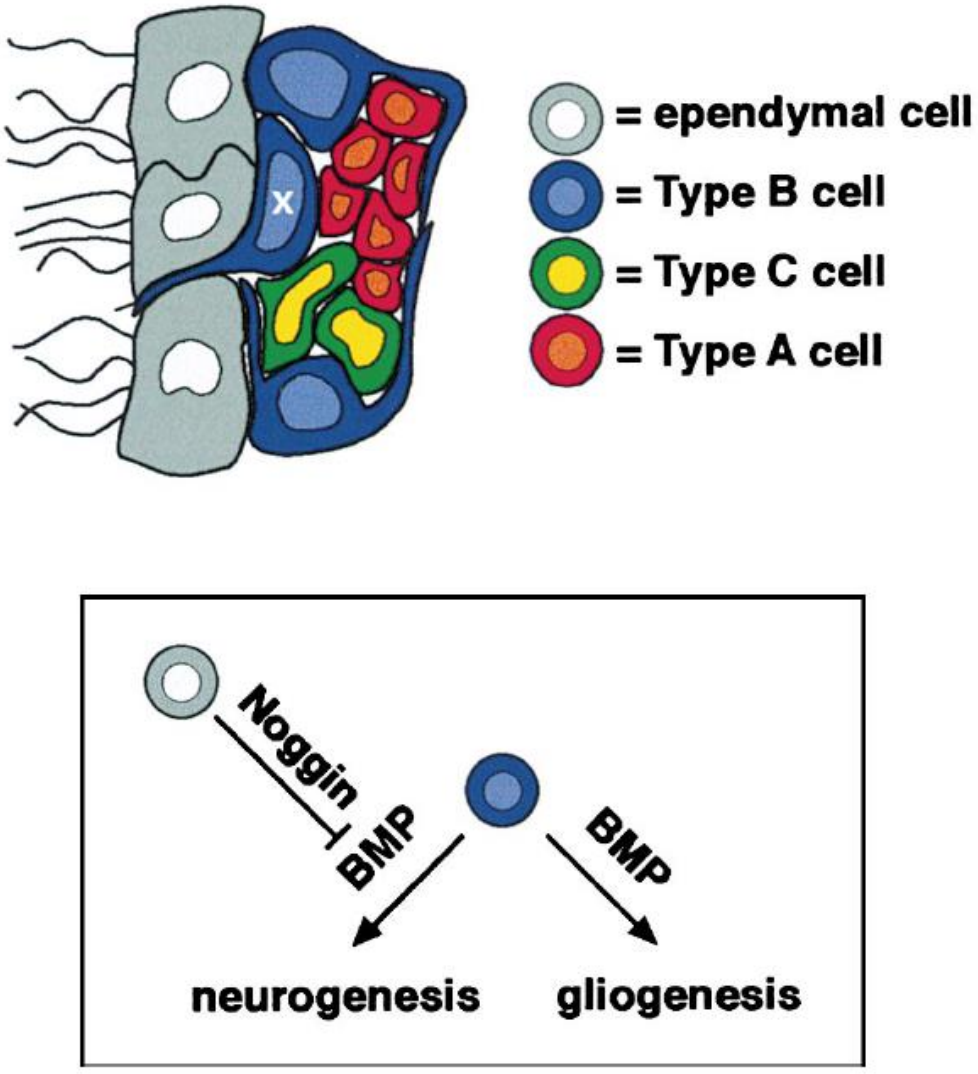


Figure 4. Schematic of SVZ Architecture and Proposed Role of Noggin and BMPs

(Top) Architecture of the SVZ. Type B cells (SVZ astrocytes) are closely apposed to ependymal cells. Some type B cells (marked with "x") extend a process between ependymal cells; these intercalating type B cells have been proposed to be cells activated for the neurogenic lineage (Doetsch et al., 1999). Clusters of type C cells are found along the chains of migratory type A cells (neuroblasts). (Bottom, in box) Proposed role of Noggin in promoting the neuronal lineage of SVZ cells. Type B cell BMP signaling blocks the neuro- genic pathway, directing type B cells to gliogenesis (right pathway). Noggin produced by ependymal cells antagonizes type B cell BMP signaling, promoting neurogenesis of SVZ cells (left pathway). The close association of type B cells and ependymal schematized above may be important for this inductive event. Used with permission[51]

BMP and Astrocytes

As briefly mentioned above, one of the main roles of BMP signaling in the postembryonic and adult brain is to promote the astrocytic differentiation of neural progenitor cells and immature astrocytes; however little is known about the mechanisms that direct this process[63]. Treatment of immature astrocytes with BMP ligands results in a more differentiated phenotype with an increased number of processes, increased expression of GFAP and decreased proliferation *in vitro*[63]. In addition, BMP2,4,5,6,7 and 10 have all been shown to promote the survival of immature astrocytes *in vitro*[63], an effect only elicited by BMPs within the TGFβ family. Astrocytes harvested from rodents at P7 highly express BMPR1A, BMPR1B, ACVR1 and BMPR2 and respond to BMP treatment canonically by phosphorylation of Smads1/5/8. Additionally, it has been shown that BMP signaling regulates the expression of VEGF in astrocytes and is necessary for the proper adhesion between astrocytes and epithelial cells for development of the blood brain barrier[64]. Clearly the role of BMP signaling during development is better established and the role of BMP signaling on the differentiated astrocytic population in the adult brain remains incompletely understood.

BMP AND CANCER

As termed by Moses and Bierie, TGFβ is the "molecular Jekyll and Hyde of cancer" having both tumor promoting and suppressing roles within tumor cells and tumor microenvironment[65]. Recently it has become clear that BMPs act in a similar manner and several reviews have detailed these findings[49,66,67]. BMPs have been shown to regulate a multitude of cancer phenotypes including proliferation, motility, invasion, metastasis, apoptosis, and epithelial-to-mesenchymal transition (EMT)[49,67]. BMPs act as both tumor suppressors and tumor promoters depending on the specific BMP ligands and receptors involved and the cancer cell type. For example, BMPs are known to both promote and inhibit proliferation in various cancer models. BMPs have been shown to enhance the proliferation of ovarian, hepatocellular carcinoma, medulloblastoma and lung cancer cells[49,68–70]. Conversely, BMPs have been shown

to inhibit proliferation in gastric carcinoma, colon cancer, and prostate cancer[66,71]. Likewise, BMP signaling can enhance or inhibit motility of cancer cells. For example, BMP signaling has been shown to enhance motility and invasion of colon, prostate and breast cancer cells[66,71]. In opposition, BMP9 has been found to suppress invasion of osteosarcoma cells[72], and BMP7 has been shown to reduce bone metastasis of breast cancer cells[73]. In our laboratory we have also observed the multifunctional role of BMP signaling in multiple cancer systems. We observed that BMPR1A receptor expression negatively correlates with progression-free survival in ovarian cancer and inhibition of BMP signaling led to a reduction in ovarian tumor sphere growth[74]. In breast cancer models, we found opposing effects based on receptor expression and cellular context. In one study, we found that deletion of BMPR2 in stromal fibroblasts led to increased tumor metastasis and increased inflammation[75]. However, in two other studies, inhibition or loss of BMPR1A resulted in less proliferative and metastatic tumors[76,77]. BMPs have also been shown to promote the differentiation of cancer stem cells in various types of cancer such as colorectal and breast cancer[66] and in the past decade, this effect has been shown to be true in GSC as well.

BMP and Gliomas

Similar to BMPs regulation of normal neural stem cells postnatally, it was discovered in 2006 by Vescovi's group[78] and further characterized in several other publications, that BMP treatment decreases proliferation and promotes astrocytic differentiation of GSC[78,79]. Piccirillo *et al* reported that treating human GSC with BMP2,5,6,7 8b and BMP4 to the greatest extent, led to decreased proliferation and increased astrocytic differentiation as shown by decreased expression of CD133, increased expression of GFAP, and a differentiated morphology consisting of flat cells with elaborated processes[78]. In a xenograft murine model, treatment of GSC with BMP4 for 48 hours prior to implantation or co-implantation with BMP4 releasing polyacrylic beads led to decreased tumor formation and increased survival[78]. In 2008, Lee *et al* published similar findings in which treatment with BMP2 or overexpression of BMPR1B reduced

tumorigenicity of human GSC through decreased proliferation and increased differentiation. Using an orthotopic xenograft model, GSC with epigenetic silenced BMPR1B resulted in decreased survival in comparison to GSC with forced expression of BMPR1B[79]. Several other studies have found similar tumor suppressing effects through decreased proliferation and increased differentiation of murine and human GSC in response to BMP signaling with the majority of the studies using BMP2,4 and 7 [80–83].

Recent studies have also shown that cells within the brain endogenously release BMP signaling molecules to modify the tumor microenvironment. Chirasani *et al* showed that untransformed neural precursor cells surround GSC and release BMP7 which acts as a tumor inhibitory molecule on GSC[84]. This phenomenon was more predominant in younger mice and was largely reduced in older mice (postnatal day 30 vs postnatal day 180)[84]. To oppose this effect, it was recently discovered that GSC express Gremlin1 (Grem1), a BMP antagonist, to inhibit endogenously expressed BMPs to maintain stemness[85]. Grem1 effectively counteracts endogenous BMPs and enhances proliferation and self-renewal while blocking differentiation in GSC. In a xenograft model, knockdown of Grem1 in the GSC population significantly increased survival, illustrating the tumor promoting role of Grem1 within the GSC population[85].

In addition to pro-differentiation effects, BMPs have been shown to sensitize GSC to chemotherapeutic treatment. Persano *et al* found that BMP2 and subsequent treatment of TMZ led to cell differentiation and decreased H1F1α expression followed by an increase in apoptosis, an effect only seen with combined BMP2/TMZ treatment[82]. Recently, a very similar study was published in which treatment of GSC with both BMP7 and TMZ led to a reduction in CD133 expression, self-renewal, migration, and significantly improved survival in a GSC tumor model over single agent treatments with BMP7 or TMZ[86].

Due to these tumor suppressive effects of BMPs on GSC, several types of therapies have been developed for use in murine models. An oncolytic vaccina virus overexpressing

BMP4 was developed and found to promote survival and prevent recurrence in xenograft models using human GBM GSC[87]. Similarly, implantable microspheres that contained and released BMP7 for 4-8 weeks decreased self-renewal of GSC while promoting differentiation, effectively reducing and delaying tumor formation in flank tumors generated from GBM GSC[88,89]. These treatments are encouraging for further development as they show vast tumor reduction and overcome some of the limitations of successful treatments such as penetration of the blood brain barrier and the short half-life of recombinant BMP proteins. Even though these therapies are promising, these studies focus solely on the effects of BMPs acting on the GSC population.

The role of BMPs on tumorigenic astrocytes has been much less studied and varying effects have been reported. In studies using human GBM cells grown as astrocytes, BMPs were reported to promote, inhibit and or have no effect on proliferation. One study using U87 cells showed growth promoting effects in response to BMP2 treatment. In this study, BMP2 treatment increased proliferation *in vitro* and increased tumor growth resulting in significantly shorter survival. In addition, suppression of BMPR1A by a micro-RNA resulted in decreased proliferation, migration and tumor growth[90]. In a series of *in vitro* experiments, Klose *et al* showed that BMP7 treatment on three human GBM derived cell lines, Gli36, U87 and A172, had contrasting anti and pro-proliferation effects, possibly resulting from the different genetic abnormalities in each cell line[91]. Finally, in the study published by Piccirillo *et al*, there was no effect on the proliferation of U87 cells treated with BMP4[78]. Similarly, Lee *et al* showed that overexpression of BMPR1B in U251 cells, a human GBM cell line, had no effect on growth kinetics *in vitro*, or on tumor growth *in vivo*[79].

Conflicting results regarding BMP signaling molecule expression and survival have also been reported. Several studies have shown that BMP receptors, ligands and ID genes are upregulated in HGG compared to low grade gliomas or normal brain tissue. In 1996, Yamada *et al* reported that the BMPRIB receptor was expressed more highly in GBM compared to lower

grade gliomas and gliosis[92]. Similarly, BMP2 was shown to be more highly expressed in GBMs compared to grade II and III gliomas[93]. Patients with high expression of BMP2 were shown to have a significantly shorter survival in comparison to those with negative expression (318 days vs. 1197 days)[93]. In addition, two separate studies have shown that ID1, ID2 and ID3 are upregulated in HGG compared to grade II gliomas, indicating increased BMP signaling in HGG[94,95]. Finally, one study has shown using microarray data that BMPR1A is more highly expressed in gliomas in comparison to normal brain tissue[90]. Conversely, several studies have shown opposing trends with downregulation of BMP molecules in HGG. In two separate studies, BMP4 expression was lower in HGG compared to low grade gliomas and non-tumor tissue at the mRNA and protein level. Additionally high BMP4 expression was associated with increased survival[96,97]. A different study found that expression of both BMPRIB and phosphorylation of Smads1/5/8 was lower in HGG compared to low grade astrocytomas and normal brain tissue. Furthermore, patients with low expression of phosphorylated Smads1/5/8 had significantly shorter survival[98] [**Table 1**].

In multiple studies, BMP molecules have been found to be associated with the PN subtype. BMP2 and BMP4 at the DNA and RNA levels were found to be increased in the PN GBM subtype based on gene expression of datasets within The Cancer Genome Atlas (TCGA) and the Chinese Glioma Genome Atlas (CCGA)[96,99,100]. According to Phillips *et al*[16], these findings would suggest that patients with a more favorable diagnosis express higher levels of BMP ligands.

BMP Signaling Molecule	Up or Downregulated in malignant gliomas (Grade III or IV)	Association with survival	Reference
BMP2	UP	Increased BMP2 associated with decreased survival	Liu 2009
BMP4	DOWN	Increased expression associated with increased survival	Bao 2013
BMP4	DOWN	Increased BMP4 associated with increased postoperative survival	Wu 2013
BMPRIB	UP	Not discussed	Yamada 1996
BMPR1B	DOWN	Not discussed	Liu 2009
p-Smad1/5/8	DOWN	High p-Ssmad1,5,8 associated with increased survival	Liu 2009
Id1	UP	Not discussed	Soroceanu 2013
Id1-3	UP	Not discussed	Vandeputte, 2002

Table 1. Published BMP molecule expression studies in gliomas

Expression levels were determined using protein or mRNA.

BMPR1A and Cancer

In studying cancer and specifically gliomas, BMPR1A is of particular interest. As mentioned previously, *Bmpr1a* is crucial for development as homozygous deletions are embryonic lethal. Constitutively active BMPR1A is also embryonic lethal, indicating the necessity for strict regulation of BMP signaling through BMPR1A[101]. Neural precursor cells continuously express BMPR1A from development through adulthood[101]. Within the adult SVZ, BMPR1A is expressed the most frequently and at the highest expression of all the BMP receptors[47]. BMPR1A was initially characterized as a tumor suppressor in the early 2000s with the discovery that germline inactivating mutations in BMPR1A, can cause patients to develop benign tumors growths in the disease known as Juvenile Polyposis Syndrome (JPS)[102,103]. JPS is characterized by the development of hamartomas, or benign tumors growing within the gastrointestinal tract[103]. JPS is an autosomal dominant syndrome which predisposes patients to gastrointestinal cancer. Approximately 20% of JPS cases are due to germline mutations in BMPR1A[104]. Similarly, conditional inactivation of BMPR1A has been shown to promote tumor growth in hair follicles, the intestines and hematopoietic stem cells[105]. However, our studies suggest that BMPR1A does not always act as a tumor suppressor. We conditionally knocked out BMPR1A in a murine model of breast cancer and found that the BMPR1A knockout cells had decreased growth *in vitro* and *in vivo* which translated into delayed tumor growth and increased survival. In addition, we found that expression of BMPR1A was correlated with decreased survival in all types of breast cancer[77]. BMPR1A has been suggested to have a tumor promoting role in gliomas as well. As mentioned previously, BMP2 and 4 are some of the primary BMP molecules used in glioma studies, and BMP2 and 4 preferentially bind to BMPR1A, suggesting an important role for BMPR1A in glioma biology. Finally, in 2014, Guo *et al* discovered that a micro RNA, miR-656 acted as a tumor suppressor by targeting and decreasing the expression of BMPR1A in glioma cells[90].

SUMMARY

As described above, BMP signaling is a critical pathway that regulates the CNS from embryonic development through adulthood. Recently it has become clear that the BMP pathway regulates glioma cells and tumor growth in several different manners. The primary finding thus far is that BMPs suppress the GSC population through promoting differentiation and decreasing proliferation. However, the role of BMP signaling in glioma initiation and progression is still largely unknown. Various results regarding BMP expression in gliomas have been published, but the vast majority of these studies focus on static markers such as BMP ligands and receptors, which are not indicative of BMP activity. In addition, the role of BMP signaling on the differentiated tumorigenic population comprising the bulk of the tumor remains largely unknown.

To begin to answer some of these questions we used two separate approaches to investigate the role of BMP signaling in HGG. The first approach was to evaluate expression levels, mutations and any correlations to survival of 90 BMP-related signaling molecules using large GBM patient datasets. Our results suggest that BMP signaling is tightly regulated within the tumor microenvironment, rarely mutated and thus likely to be critical to tumor cell survival. The second approach we used was to investigate the role of BMP signaling in the differentiated cells comprising the bulk of the tumor. Our findings demonstrate that BMP signaling is a tumor promoter in the context of tumorigenic astrocytes. Our studies indicate that BMP signaling is present and active in the vast majority of HGG cells, and that BMP signaling regulates tumorigenic cells differently depending on the cellular context. The results presented in this thesis provide insight into relatively unstudied areas of HGG biology and suggest that inhibition of the BMP pathway may be a novel and promising treatment for these devastating tumors.

CHAPTER II: GENOMIC ANALYSIS OF THE BMP FAMILY IN GLIOBLASTOMAS

This is a pre-copyedited, author-produced, adapted version of an article accepted for publication in Translational Oncogenomics following peer review. The version of record is: Hover, LD., Abel, TW., & P. Owens. (2015) Genomic Analysis of the BMP Family in Glioblastomas. Transl Oncogenomics, 15; 7:1-9.

INTRODUCTION

GBMs are well known for their histologic heterogeneity and one of the primary challenges in finding effective therapies is due to the high level of heterogeneity found within GBMs. Recently with the rapid advancement of sequencing technology and accumulation of genomic data, an emphasis has been placed on understanding these tumors at the molecular genetic level. In 2008, GBMs were the first type of cancer to be profiled by TCGA which led to the identification of core pathways and alterations found in the majority of GBMs [12]. Extensive profiling of GBMs has expanded to include epigenomic, transcriptomic and proteomic analyses collecting data on the DNA sequence of the whole-genome and exome, DNA copy-number, mRNA sequencing and expression data, CpG DNA methylation, miRNA expression, protein expression and corresponding clinical characteristics [12,106,107]. These analyses of GBMs have provided critical insight furthering our understanding of GBMs by identifying novel mutated genes, patterns of mutation, chromatin modifications, recurrent gene rearrangements, enhancing our understanding of the underlying molecular biology of GBMs. Through these studies it has become clear that alterations, including mutations in RTKs, PI3K, PTEN, NF1, and IDH proteins are central to GBM biology[10,12].

Furthermore, these analyses have segregated GBMs into subclasses based on gene expression analysis, revealing enriched gene expression signatures within each subgroup as discussed in the introduction. Among several studies, two dominant groups have been established, termed the Proneural (PN) and the Mesenchymal (MES) group, reflecting the

dominant gene expression patterns in each group [108]. These subgroups show gene expression patterns similar to those found within neural lineages [16]. In 2006, Phillips *et al*. likened the gene expression signatures to various stages of neurogenesis in the adult forebrain. Thus, gene expression patterns of MES tumors are akin to that of undifferentiated neural stem cells and PN gene expression is similar to neuroblasts or immature neurons[16]. Importantly, the gene expression within these tumors is mutually exclusive, effectively establishing two separate types of GBMs. Although Phillips *et al*. reported increased survival in PN patients, other studies have not shown associations with survival in any subtypes[108]. These data suggest that a better understanding of the pathways regulating neural development is crucial to understand the underlying signaling pathways and networks that give rise to GBM subtypes.

As one of the primary pathways involved in neural development, the BMP pathway is a critical pathway to understand in the development of gliomas and GBM subclasses. Although many studies have shown the tumor-suppressive effects of BMP signaling on GSC, little has been reported about BMP signaling in the context of GBM genomics. To gain a better understanding of BMP signaling in human GBMs, we queried BMP pathway alterations at the genetic level to assess how the BMP signaling network is altered in patient samples. We used publicly available data compiled and analyzed through The Cancer Genome Atlas (TCGA) to examine gene expression and mutations and the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) to analyze associations between gene expression and patient survival. We expanded our search beyond the immediate BMP family and analyzed 90 genes within the TGFβ family including receptors, ligands, inhibitors and downstream targets known to interact directly with the BMP pathway [**Table 2**]. To identify genes within the TGFβ family that show either increased or decreased expression in GBMs we accessed TCGA data using the cBio portal for Cancer Genomics maintained through the Memorial Sloan Kettering Cancer Center [109,110]. We analyzed the mutational status and mRNA expression of 598 individual GBM samples using the TCGA provisional dataset.

BMP Ligands	Receptors	Co-Receptors	Intracellular Mediators	Modulators	Modulators (con't)	IDs	TGFB ligands	Activin	GDFs	Misc
BMP2	*ACVR1*	*BAMBI*	*SMAD1*	*AMN*	*NBL1*	*ID1*	*TGFβ1*	*AMH*	*GDF1*	*ARTN*
BMP3	*ACVR1B*	*BMPER*	*SMAD2*	*CER1*	*NOG*	*ID2*	*TGFβ2*	*GDNF*	*GDF3*	*BMP1*
BMP4	*ACVR1C*	*TGFBR3*	*SMAD3*	*CHRD*	*SOST*	*ID3*	*TGFβ3*	*INHBA*	*GDF5*	*HFE2*
BMP5	*ACVR2A*	*TMEFF1*	*SMAD4*	*CHRDL1*	*SOSTDC1*	*ID4*		*INHBB*	*GDF6*	*NEO1*
BMP6	*ACVR2B*	*TMEFF2*	*SMAD5*	*CHRDL2*	*TSKU*			*INHBC*	*GDF7*	*NRTN*
BMP7	*ACVRL1*		*SMAD6*	*DAND5*	*TWSG1*			*INHBE*	*GDF9*	*PSPN*
BMP8B	*AMHR2*		*SMAD7*	*ENG*	*USP9X*			*LEFTY1*	*GDF11*	*RGMA*
BMP10	*BMPR1A*		*SMAD9*	*FST*	*VWC2*			*LEFTY2*	*GDF15*	*RGMB*
BMP15	*BMPR1B*		*SMURF1*	*FSTL1*				*NODAL*	*MSTN*	
GDF2	*BMPR2*		*SMURF 2*	*FSTL3*						
	TGFβR1			*GREM1*						
	TGFβR2			*GREM2*						

Table 2. List of 90 genes related to the TGFβ family that was analyzed at the genomic level

Genes were analyzed at the mRNA level for both alterations and any associations with survival in GBM patients.

mRNA expression is considered to be significantly up or downregulated if the expression is above or below two standard deviations of the mean determined from Agilent microarray data. We found that out of our query of 90 genes, 44 of those were altered in 5% or more of patients [**Table 3**].

In parallel, we analyzed the association between the mRNA expression of these 90 genes and patient survival using a dataset of 181 GBM patients available using REMBRANDT maintained by the National Cancer Institute (NCI) [111]. We investigated if two-fold up or downregulation of the mRNA levels analyzed using the U133 2 Plus mRNA expression chips (Affymetrix) is associated with increased or decreased overall survival. mRNA increases or decreases in expression is determined in comparison to non-tumor pooled samples[112]. Out of our set of 90 genes, 19 genes were significantly associated with either increased or decreased overall survival [**Table 4**].

After examining BMP signaling molecules in all GBMs, we focused on associations with the GBM molecular subgroups. Previous studies have shown that BMP2 and 4 are associated with the PN glioma subtype[96,99] and we sought to determine if other members of the BMP family are also associated with the PN subtype. To investigate these associations, we used TCGA data to examine the gene expression of 620 patients classified into subgroups. Here we show how mRNA expression is altered in GBM samples and how that is associated with patient survival highlighting both known and novel associations between BMP signaling and GBM biology.

Genes	Percent altered in GBMs (598)	Number of patients with mutated gene	Number of patients with mRNA upregulation (>2 Std. dev)	Number of patients with mRNA downregulation (>2 Std. dev)
BMP Ligands				
BMP7	5.2	0	15	16
BMP8B	10.2	0	9	52
Receptors				
BMPR1A	5.7	0	1	33
TGFBR1	7.7	2	6	38
TGFBR2	5	2	15	11
ACVR1	6	0	26	10
ACVR1B	8	0	7	41
ACVRL1	7.5	1	13	31
ACVR2B	6	0	29	6
ACVR2A	5	0	26	2
AMHR2	7	1	11	31
Co-receptor				
BMPER	5.5	1	32	0
Intracellular Mediators				
SMAD1	6.5	0	17	22
SMAD2	7.2	0	22	21
SMAD3	6.2	0	13	24
SMAD4	7.7	1	22	23
SMAD6	6.2	0	12	25
SMAD9	7.2	0	12	31
SMURF2	6	0	15	21
IDs				
ID2	6.2	0	20	17
ID3	5.2	0	3	28
Modulators				
CHRD	8.9	1	7	46
AMN	8.7	0	9	43
ENG	6.7	1	14	25
FSTL1	6	1	11	24
SOST	6	0	19	17
TWSG1	5.7	0	10	24
GREM2	5	0	16	14
TGFβ Ligands				
TGFβ1	5	0	14	16
TGFβ2	5	0	12	18
TGFβ3	6.4	0	14	24

Table 3. Genes altered in ≥5% of human GBMs

Genes	Percent altered in GBMs (598)	Number of patients with mutated gene	Number of patients with mRNA upregulation (>2 Std. dev)	Number of patients with mRNA downregulation (>2 Std. dev)
Activins				
NODAL	10.7	0	62	2
AMH	8.4	0	8	42
GDNF	7.2	2	12	29
LEFTY2	5.9	2	16	17
INHBC	5.4	0	16	16
INHBE	5.7	1	26	7
Misc.				
NRTN	9.2	0	11	44
BMP1	7	0	15	27
ARTN	6.4	0	12	26
PSPN	6.2	0	19	18

Table 3. Genes altered in ≥5% of human GBMs

All data were collected by the TCGA and analyzed using the cBio Portal for Cancer Genomics. Our dataset consisted of 598 individual GBM samples, and we analyzed 90 genes. For each gene altered in more than 5% of GBMs, we determined in how many tumor samples the gene was mutated and mRNA expression was upregulated or downregulated. mRNA upregulation and downregulation is considered to be >2 standard deviations from the mean expression.

Gene	mRNA Expression	Log rank p-value	Associated with Increased or Decreased Survival:	Average Overall Survival (months)	Number of Patients with mRNA alteration
GDNF	Up-Regulated	0.01	Increased	26.3	46
BMP8B	Down-Regulated	0.02	Increased	29.1	19
GDF5	Up-Regulated	0.04	Increased	24.9	36
BMP5	Up-Regulated	0.05	Increased	42.6	7
CHRDL1	Up-Regulated	0.05	Increased	39.2	6
RGMA	Down-Regulated	0.05	Increased	72.9	2
BMP6	Down-Regulated	0.000009	Decreased	8.9	12
GDF15	Down-Regulated	0.000065	Decreased	2.2	1
ACVR1B	Down-Regulated	0.0014	Decreased	15.5	82
GDNF	Down-Regulated	0.01	Decreased	14.0	32
NBL1	Down-Regulated	0.01	Decreased	16.6	110
NRTN	Down-Regulated	0.01	Decreased	13.5	33
SMAD6	Up-Regulated	0.01	Decreased	10.4	10
SOST	Up-Regulated	0.01	Decreased	14.0	45
TSKU	Up-Regulated	0.01	Decreased	17.8	152
FSTL1	Up-Regulated	0.02	Decreased	18.1	154
INHBE	Down-Regulated	0.02	Decreased	14.7	39
SMAD1	Up-Regulated	0.02	Decreased	15.4	65
TWSG1	Up-Regulated	0.02	Decreased	17.5	121

Table 4. mRNA expression of genes associated with a significant increase or decrease in overall survival

Using the publicly available database REMBRANDT, we were able to determine in which genes upregulation or downregulation of mRNA expression is associated with significant ($p<0.05$) increased or decreased patient overall survival. Upregulation and downregulation are considered to be more than twofold change from expression in nontumor pooled samples. Log-rank P-values were calculated using the Mantel–Haenszel procedure. For each gene that was associated with overall survival, we determined the number of patients that expressed the alteration and calculated the average overall survival in months. Our dataset consisted of 181 GBM patients with a total overall survival of 19.6 months.

MATERIALS & METHODS

TCGA Analysis

cBioPortal for Cancer Genomics: Gene mutation status and mRNA expression were analyzed using publically available data obtained through the cBio Cancer Genomics Portal ((http://www.cbioportal.org/public-portal/ [accessed May 2014][109,110]. We selected the Glioblastoma Multiforme (TCGA, Provisional) dataset from the Brain CNS Cancer Study category. Within the Genomic Profiles options we selected mutations and mRNA expression data from Agilent microarray data using a z-score threshold of 2.0. Z scores were determined by comparing the mRNA expression of each tumor sample to the mean expression value of all tumors that are diploid for the gene of interest. At the time of access, there were 598 patients available within the "All Tumors" GBM TCGA provisional dataset.

Subtype Analysis

mRNA expression values were integrated from 3 independent gene expression platforms: Affymetrix HuEx array, Affymetrix U133A array and Agilent 244K array. All data was collected through the TCGA. At the time of access, there were 202 subtyped patients available [November 2012]. Significance was determined by ANOVA followed by post-hoc t-tests.

Survival Analysis

REMBRANDT

Microarray gene expression and survival data was acquired from the publically available NCI Repository for Molecular Brain Neoplasia Data (REMBRANDT) database (https://caintegrator.nci.nih.gov/rembrandt/home.do [accessed May 2014][112]. To analyze associations with survival we selected the graph format: Kaplan-Meier survival plot for Gene Expression Data. We restricted the analysis to GBM patient samples. At the time of access there were 181 GBM samples. Gene expression was determined from U133 2 Plus mRNA expression chips (Affymetrix). Up and down regulation were determined as 2-fold or greater difference than pooled non-tumor samples. Log-rank p values were calculated using Mantel-

Haenszel procedure to determine significance between groups of samples stratified by levels of gene expression[111]. Gene associations with overall survival were compared to the overall survival for all 181 GBM patients. The average overall survival for all 181 patients is 19.6 months. To determine average overall survival we reviewed the clinical reports of patients segregated by gene expression and calculated the mean overall survival.

cBio Cancer Genomics Portal

Survival analysis within the PN subtype was conducted using the cBio Cancer Genomics Portal using the Glioblastoma Multiforme TCGA Nature 2008 dataset from the Brain CNS Cancer Study category. ((http://www.cbioportal.org/public-portal/). Within the Genomic Profiles options we selected mutations and mRNA expression data from Agilent microarray data using a z-score threshold of 2.0. Z scores were determined by comparing the mRNA expression of each tumor sample to the mean expression value of all tumors that are diploid for the gene of interest. There were 56 patients within the PN subtype in the dataset at the time of access [August 2015]. Survival curves were generated using Kaplan-Meier estimate and significance was determined using a log-rank test[109,110].

RESULTS

Genes expression analysis

First we analyzed the mutational status and mRNA expression of 598 individual GBM samples using the TCGA provisional dataset. mRNA expression is considered to be significantly up or downregulated if the expression is above or below two standard deviations of the mean determined from Agilent microarray data. We found that out of our query of 90 genes, 44 of those were altered in 5% or more of patients [**Table 3**]. Next we investigated if two-fold up or downregulation of the mRNA levels analyzed using the U133 2 Plus mRNA expression chips (Affymetrix) is associated with increased or decreased overall survival in a dataset of 181 GBM patients. mRNA increases or decreases in expression is determined in comparison to non-

tumor pooled samples[112]. Out of our set of 90 genes, 19 genes were significantly associated with either increased or decreased overall survival [**Table 4**].

Based on our cumulative results, we found that 4 genes: *BMP8B*, *ACVR1B*, *SMAD1*, and *NRTN* are both altered in more than 5% of patients and show an association with survival. The role of these four genes in relation to GBMs is largely unknown. Here we report the known function of each gene and its relation to gliomas.

BMP8B (*OP-2*) first described by Ozkaynak *et al*. in 1992 was found to be a member of the TGFβ family identified through cDNA library screenings. BMP8B is expressed early in embryogenesis [113]. Although little is known about this protein in relation to gliomas, BMP8B treatment has been shown to decrease proliferation of glioma stem cells [78]. Interestingly, the mRNA expression available through the TCGA shows that *BMP8B* mRNA expression is downregulated in 52 patients and upregulated in only 9 patients. Therefore, downregulation of *BMP8B* mRNA expression accounts for 85% of total *BMP8B* alterations. Within the REMBRANDT GBM dataset, downregulation of *BMP8B* correlates with increased patient survival (p=0.02). The 19 GBM patients within this data set with downregulated *BMP8B* had an average overall survival of 29.1 months.

ACVR1B, Activin A type IB receptor (ALK4), is part of the activin subfamily within the TGFβ family. Activins are members of the TGFβ family known for their role as growth and differentiation factors. *ACVR1B* was originally discovered using a sequence-based polymerase chain reaction (PCR) approach by ten Dijke *et al*. in 1993. *ACVR1B* mRNA is ubiquitously expressed in all tissues, most strongly in the kidneys, pancreas, brain, lung, and liver [114]. *ACVR1B* mutations have been identified and found to have varying effects in several types of cancer. In prostate cancer, Nomura *et al*. showed that cell lines with constitutively active AVCR1B had increased migratory ability aiding in EMT. In a neuroblastoma cell line, Suzuki *et al*. showed that ACVR1B specific activin signaling induced neuronal differentiation [115]. Little is

known about the role of *ACVR1B* on gliomas. The TCGA data we compiled shows that *ACVR1B* expression is frequently decreased (41 out of 49 alterations, 85%) when altered in GBMs. Furthermore within the REMBRANDT data set, downregulation of *ACVR1B* was associated with decreased survival (p=0.0014). The 82 patients with *ACVR1B* downregulation had an average overall survival of 15.5 months compared to the average overall survival of the 181 patients at 19.6 months.

SMAD1 belongs to the Smad family, a family of proteins that serve as the signal transducers for canonical BMP and TGFβ signaling. While these Smad genes were originally discovered and understood in Drosophila and C. elegans, *SMAD1*, the human homolog, was first discussed and cloned in 1996 [116,117]. This protein is activated through BMP receptor-phosphorylation leading to downstream transcriptional regulation. In gliomas Liu *et al*. showed that phospho-SMAD1 is expressed at lower levels in glioma samples in comparison to normal brain tissue[98]. Additionally, it was found that patients with a high ratio of phosphorylated SMAD1/5/9 to SMAD1 had increased survival, demonstrating that increased SMAD1 activation is beneficial to patient survival [98]. This suggests that increased BMP signaling and increased SMAD1 phosphorylation provides a survival benefit. Using REMBRANDT we observed that increased expression of *SMAD1,* found in 65 patients, is associated with decreased survival (p=0.02) when compared to the total overall survival of REMBRANDT GBM patients (15.4 months vs 19.6 months). The mRNA expression data available through the TCGA shows that *SMAD1* mRNA is equally up and down-regulated within *SMAD1* altered GBM samples (17 and 22 patients show upregulation and downregulation respectively). As described above, analyzing the expression of *SMAD1* in parallel with the phosphorylation of SMAD1 will be more informative in regards to GBMs and patient survival.

NRTN, Neurturin is a neurotrophic factor serving to promote the survival of various neuronal populations [118]. *NRTN* was first isolated in 1996 by Kotzbauer *et al*. after being

identified by its ability to support sympathetic neurons in culture. *NRTN* is closely related to the glial cell line-derived neurotrophic factor (*GDNF*), both known as TGF-beta-related neurotrophins (TRNs). TRNs belong to the TGFβ family based on structural similarity and the presence of conserved cysteine residues, yet TRNs share less than 20% amino acid sequence similarity to other TGFβ family members [118,119] Little is known about the role of *NRTN* in gliomas however it has been reported that *NRTN* promotes pancreatic cell aggressiveness through both proliferation and invasion [120]. The mRNA expression data we examined shows that *NRTN* expression is frequently downregulated when altered in GBMs (44 out of 55 alterations). Using the REMBRANDT dataset NRTN downregulation is present in 33 patient samples and is associated with decreased survival with the average survival of 13.5 months (p=0.01).

In addition to the genes described above, we chose to further investigate five genes: *SMAD4, BMPR1A, BMP5, ID1 AND GREM1. SMAD4*, *BMPR1A* and*ID1* were primarily selected due to their crucial role in mediating canonical BMP signaling. We selected *BMP5* because we found 4% of all GBM patients to show upregulation of *BMP5* which was also found to be associated with increased survival. Finally, *GREM1* was chosen because of the recently published finding showing that glioma stem cells secrete *GREM1* to promote tumorigenesis through inhibition of BMP signaling [85].

SMAD4*,* this member of the Smad family is of particular interest as it is a central regulator of both TGFβ and BMP signaling. *SMAD4* was originally discovered as a tumor suppressor in pancreatic cancer in 1996 by Hahn *et al*. after it was found to be homozygously deleted in 25 of 84 pancreatic tumors[121]. *SMAD4* has been shown to be involved in many other types of cancer primarily through chromosome deletion. *SMAD4* has been shown to be inactivated in 48% of pancreatic tumors but is inactivated in less than 10% of tumors in other types of cancer [122]. These deletions have been described in colon cancer [123] head and neck

squamous cell carcinoma [124], breast and ovarian [122]. In gliomas, He *et al*. showed that *SMAD4* expression is reduced in all gliomas in comparison with normal brain tissue with the lowest expression in high grade gliomas[125]. In addition He *et al*. found that the loss of *SMAD4* is correlated with poor survival [125]. In our analysis we found *SMAD4* to be dysregulated in about 8% of the GBM dataset queried with equally distributed up and downregulation (22 and 23 respectively). Furthermore, our analysis showed no significant association between survival and up or downregulation of *SMAD4* mRNA expression (p=0.68, p=0.73 respectively).

***BMPR1A*,** Bone morphogenetic protein receptor IA, (*ALK3*), is a type I receptor in the TGFβ family. Using PCR technology ten Dijke *et al*. discovered *BMPR1A* in 1993 based on sequence homology to the human activin receptor type II and a type I-like TGFβ receptor in C. elegans [114]. Mutations in *BMPR1A* have been shown to cause juvenile polyposis in many patients, a condition characterized by benign growth within the gastrointestinal tract [102–104,126–128]. Guo *et al*. in 2014 showed that the micro-RNA, miR-656 acted as a tumor suppressor in gliomas by specifically repressing expression of *BMPR1A* [90]. Our analysis using TCGA data shows that *BMPR1A* mRNA expression is altered in approximately 5.7% of GBMs with the vast majority of those being downregulated (33 out of 34 alterations). Within the REMBRANDT dataset there were no GBM patient samples available with downregulation of *BMPR1A*. We found no association between upregulation of mRNA expression of *BMPR1A* and survival (p=0.23).

***BMP5*,** bone morphogenetic proteins were originally identified by Urist in 1965 due to their ability to induce endochondral osteogenesis *in vivo* at an extraskeletal site [36]. *BMP5* based on sequence homology is in a subgroup with BMP 6, 7 and 8b [37]. In adrenocortical carcinoma and pancreatic cancer, expression of *BMP5* was found to be downregulated [71,129]. *BMP5* was shown to inhibit cell proliferation yet increase migration and invasion in pancreatic cancer cell lines [71]. *BMP5* has been shown to decrease proliferation of glioma stem cells [78]. Our mRNA expression data show that *BMP5* is altered in 4.8% of GBMs. 86% of those alterations were

due to upregulation of *BMP5* (25 out of 29 alterations), and we found upregulation of *BMP5* to be associated with increased survival of GBM patients (p=0.05). The 7 patients available within the REMBRANDT database with upregulation of BMP5 had an average overall survival of 42.6 months, more than twice the average survival.

***ID1*,** inhibitor of DNA-binding 1, is a key transcriptional regulator that is a specific downstream target of active BMP signaling. ID proteins inhibit the binding of DNA to other transcriptional factors by binding to the helix-loop-helix motif of transcriptional factors. *ID1* was isolated from human fibroblasts in 1994 by Hara *et al*. [130]. *ID1* has been shown to regulate the cell cycle and differentiation of cells in a wide variety of cell types [130–133] including normal neural and glioma cells. In 2009 Nam and Benezra showed that *ID1* and *ID3* are required to maintain self-renewal of the type B adult neural stem cells. In addition it was shown that *ID1* can be used to identify type B neural stem cells within the stem cell niches of the brain and that ID1 protein levels decrease during the process of cell differentiation [134]. However, the role of *ID1* in GBM biology has proven to be very complex. *ID1* expression has been shown to be upregulated in human gliomas and murine experimental models of glioma [95,135]. In 2013 Soroceanu *et al*. showed that *ID1* levels correlate with tumor histopathologic grades and tumor cell invasiveness *in vitro* and that knockdown of *ID1* increased survival in an orthotopic model of GBM [94]. Contrastingly, Barrett *et al*. showed using a murine model of HGG that glioma cells with both high and low levels of *ID1* are tumorigenic and surprisingly the low *ID1* expressing cells formed tumors more rapidly and with higher penetrance [136]. According to our analyses *ID1* expression is altered in approximately 4.5% of GBMs, equally up and downregulated in the TCGA sample population (13 patients each). In the patient sample available through REMBRANDT, the five GBM patients with low *ID1* mRNA expression had significantly decreased survival with an average overall survival of 8.5 months (p=0.01). *ID1* plays a prominent role in regulating both normal and tumor cells and warrants further investigation.

GREM1, Gremlin 1, is an antagonist of BMP signaling. GREM1 is a secreted molecule that binds to BMP ligands to prevent them from binding to their receptors. *GREM1* was isolated in 1998 by Hsu *et al*. by cloning the human homolog of the gremlin gene in Xenopus [137]. *GREM1* is a crucial mediator of development by inhibiting BMP signaling [138]. We chose to specifically investigate the expression of *GREM1* due to the recent publication: "Glioma cancer stem cells secrete Gremlin1 to promote their maintenance within the tumor hierarchy" by Yan *et al*. in 2014. This publication shows that GREM1 is endogenously expressed by GSC to protect their self-renewal ability and stem-like state from the pro-differentiation effects of BMP signaling. GREM1 secretion is thought to contribute to treatment resistance through maintaining cellular proliferation, cellular hierarchies within the tumor as well as increasing resistance to differentiation therapy [85]. Our analysis shows that *GREM1* is altered in approximately 3.8% of GBMs (23 patients), all of which are mRNA upregulations. Interestingly, using the REMBRANDT dataset, mRNA upregulation was present in 12 of the 181 patients showing enhanced survival with an average survival of 40 months, which is more than twice the average survival (p=0.06).

BMPs and Proneural association

For our analysis we focused on significant differences in expression between the PN and MES subtype as those are the most well-defined subgroups. We analyzed the expression of primary BMP ligands, receptors, inhibitors, Smads and ID genes including: *ID1, ID3, Noggin, BMP2,4,6, SMAD6,7, BMPR1A, BMPR1B* and *BMPR2* and determined any significant associations with the PN or MES subtype.

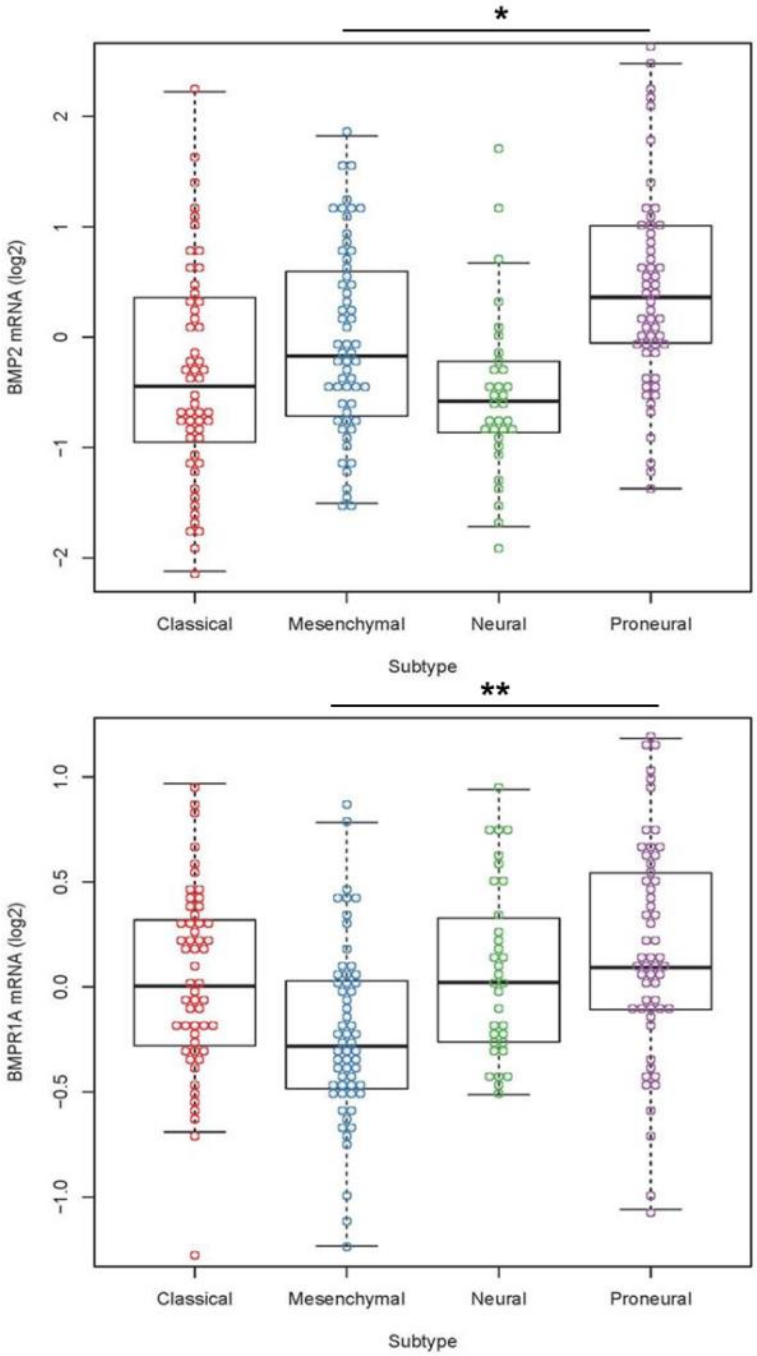


Figure 5. mRNA expression of BMP signaling components are upregulated in the PN subtype compared to the MES subtype.

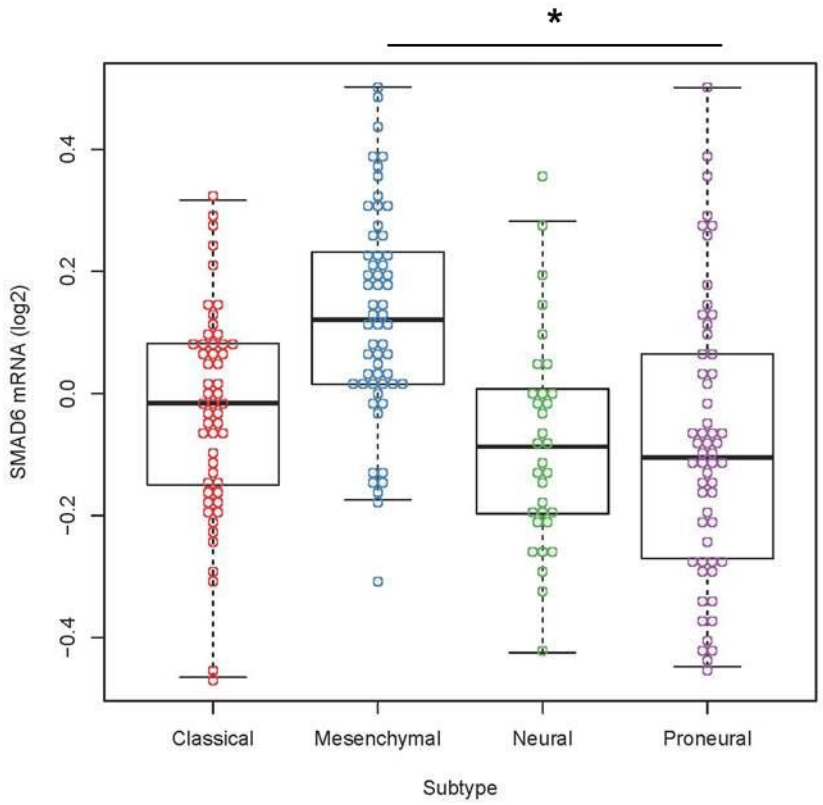


Figure 5. mRNA expression of BMP signaling components are upregulated in the PN subtype compared to the MES subtype

Box plots comparing mean gene expression levels for BMPR1A, BMP2 and SMAD6. Both the receptor and ligand mRNA expression are upregulated in PN tumors compared to MES tumors. SMAD6 mRNA is upregulated in MES tumors compared to PN tumors. mRNA expression levels are integrated from 3 independent gene expression platforms: Affymetrix HuEx array, Affymetrix U133A array, and Agilent 244K array. All data were collected from TCGA data portal. *p<0.05, **p<0.01

Using expression data compiled by the TCGA, we found that *BMP2* and *BMPR1A* are expressed more highly in the PN subtype [**Figure 5**]. Unlike other studies, we did not find the expression of *BMP4* to be associated with the PN subtype. Interestingly, we found that *SMAD6* expression was significantly higher within the MES subtype, suggesting suppression of BMP signaling within the MES subtype [**Figure 5**]. No other molecule analyzed was associated with or overexpressed within a subgroup.

Upon further analysis of *BMP2* expression within the PN tumor group, we found that patients with two-fold or higher increase in *BMP2* mRNA had a significantly longer median survival of approximately 47 months compared to approximately 11 months in patients without *BMP2* upregulation, $p<0.01$ [**Figure 6**]. Due to the small number of patients available for survival analysis, we were not able to determine if *BMPR1A* or *SMAD6* was associated with survival in the PN tumor group.

CONCLUSIONS

The BMP signaling pathway is a complex network of receptors, ligands, and antagonists all of which may be capable of dynamically impacting GBM growth, maintenance and progression both positively and negatively. As with TGFβ signaling, it appears as though BMP signaling can modulate tumor growth and maintenance in various ways and most likely plays a context dependent role in GBM tumor growth. Our analysis shows that upregulation and downregulation of ligands, receptors and intracellular modulators are associated with both increased and decreased survival. Similarly, previous reports have shown conflicting data regarding the expression of various BMP signaling molecules and survival in human GBM [92,93,96]. To better understand this pathway and how we may be able to exploit the signaling for novel drug treatments, we examined patient samples at the genetic level to explore the BMP pathway in GBMs.

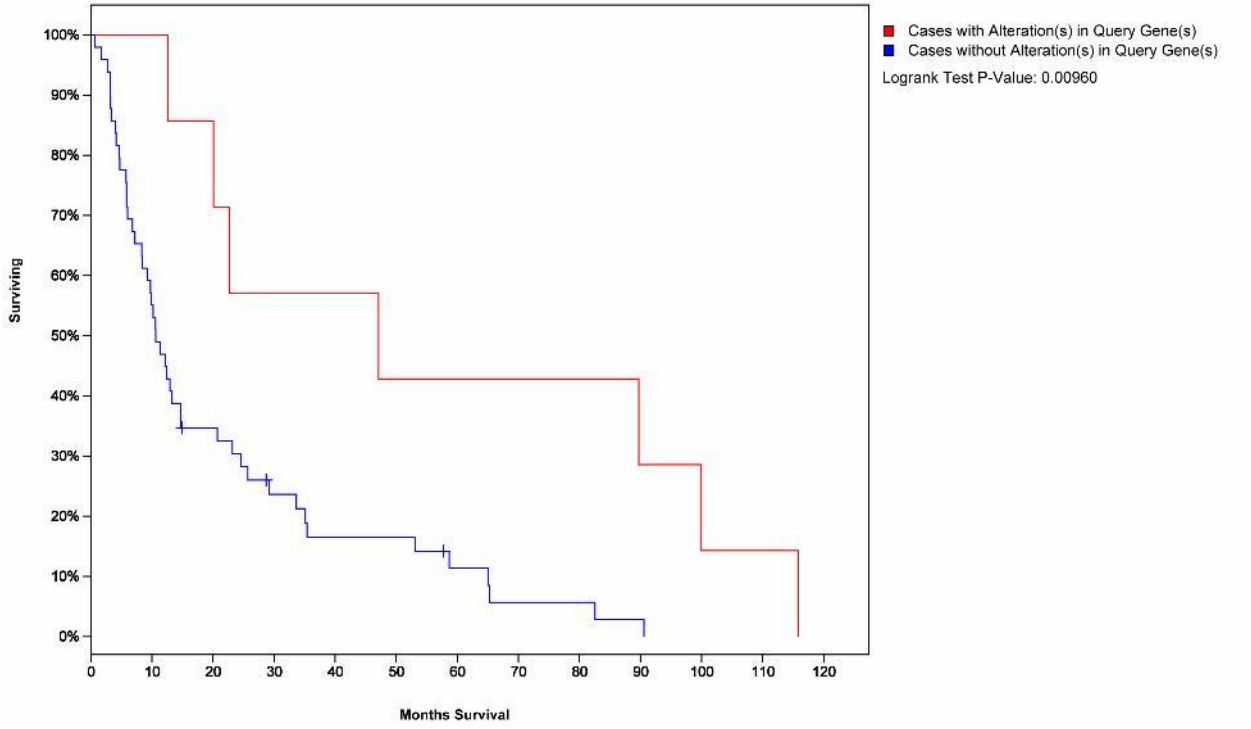


Figure 6. Survival curve showing patients with increased survival time in months for patients with increased expression of BMP2 in the PN GBM subtype

In a dataset of 56 PN patients, those with ≥ 2 fold upregulation of BMP2 mRNA have a median survival of 47 months compared to a median survival of 10.6 months in those without upregulation of *BMP2*. mRNA expression levels are integrated from 3 independent gene expression platforms: Affymetrix HuEx array, Affymetrix U133A array, and Agilent 244K array. All data was collected and processed using the cBioPortal for Cancer Genomics[109,110].

BMP signaling is required for development and the regulation of neural cells. As reviewed by Guang-Quan Zhao *et al*. in 2002, targeted mutagenesis of BMP ligands, receptors and other pathway modulators has shown that BMP signaling is involved and critical to almost all aspects of development [53]. Given the high importance of BMP signaling, especially with a focus on neural cells, we hypothesize that BMP signaling is likely necessary for the survival of tumor cells and is very tightly regulated within the tumor environment, which may explain why the pathway is attenuated and not genetically deleted within GBMs. In support of our hypothesis, we observed that within the 598 tumors surveyed using the TCGA GBM provisional dataset, none of the genes queried are altered in more than 15% of the tumors and mutations are exceedingly rare. The majority of the genes examined are not altered in more than 5% of GBMs. The alterations seen primarily occur in non-overlapping tumors, indicating that compensation or redundancy in the pathway is possible and perhaps necessary for survival of tumor cells in GBMs.

However, we may be able to exploit this importance to tumor cell survival in a way to benefit patients. As shown in xenograft models there have been several forms of BMP treatments that have shown increased survival and decreased tumor growth which should be considered for development in the clinic [78,79,81,87]. More studies need to be completed to show that increased levels of BMP signaling *in vivo* at high doses do not act as a tumor promoters. Understanding the larger impact of BMP treatment on the bulk of the tumor and the microenvironment is crucial prior to the development of BMPs into the clinic. As our analysis shows, increased expression of molecules involved in BMP signaling is not always associated with increased survival.

One reason that we may not see consistent expression patterns of BMP molecules may be due differences in expression within glioma subtypes. Phillips *et al* likens the phenotypes of each subtype to different stages in neurogenesis of the adult forebrain[16]. Based on these

expression and phenotypic differences in subtypes, it is likely that the actions of BMPs differ depending on the glioma subtype. In concordance with previous studies, our results show that certain BMP molecules are expressed more highly in the PN subgroup[96,99]. Within the PN group we found that overexpression of *BMP2* was associated with a significantly longer survival suggesting that BMP signaling may confer a survival advantage within PN tumors. Previously increased *BMP2* expression was associated with significantly shorter survival when analyzed across all gliomas [93] supporting our hypothesis that BMP signaling impacts tumors differently depending on the subtype and phenotype of the tumor cells. Contrary to a previous study, we did not find that *BMP4* was overexpressed in the PN tumors[96]. This discrepancy may be due to our limited sample size. Bao *et al* found using 422 glioma samples that *BMP4* was more highly expressed in PN tumors[96]. In addition, the authors showed that *BMP4* expression was associated with increased survival in GBM patients [96]. BMP signaling and associations with subtypes will become more apparent as a larger number of samples are available and more in depth studies can be done. Another limitation to our understanding of these subtypes is the lack of animal models that accurately represent each subtype. As more studies are done and more accurate models are developed we will begin to understand the mechanisms underlying the formation and maintenance of these subgroups and specifically how the BMP pathway is involved.

The nature of genomic studies allows for a network view of individual pathways and uncovers genes within the pathway that may have remained unnoticed. Our analysis primarily serves as a guide for future research, emphasizing genes that are altered in a significant number of patients and are associated with survival. Here we highlight several genes largely unstudied in the context of GBMs which are both altered in a high percentage of patients and have associations with survival. Our analysis of 90 genes related to the BMP pathway provides

a network view of the alterations occurring within the pathway to complement the many single-gene research studies that have been done.

However, there are several factors that should be considered when interpreting this data. As with the vast majority of genomic studies, the data collected presents a snapshot of the tumor. GBMs are known for their intratumoral heterogeneity, therefore it is likely that the gene expression and gene alterations differ throughout the tumor and differ over time during tumor initiation and progression. From these studies it is impossible to know when these mutations and alterations arose during the progression of the tumor. It is unknown which of the alterations reported were present in the initial tumor promoting cells and which have been acquired in response to the selective pressures of the tumor microenvironment, treatments and tumor resections. Johnson *et al*. demonstrated this phenomena showing vast differences in the genomic alterations and mutations present in initial and recurrent tumors [139]. For example, Johnson *et al*. showed that *SMADs 4,6,7* and *9* were mutated in recurrent tumors and not initial tumors, suggesting that alterations in Smads may not be involved in driving the initial tumor formation [139]. Additionally, our analysis is based off limited patient data which may contain diverse genetic and treatment variables that in the future could be important in determining appropriate therapies. Finally, the results need to be considered in context with the microenvironment including immune infiltrates, metabolic changes and angiogenesis

Given these caveats our ultimate goal in sharing these findings is that our analysis will guide future studies to a novel and more complete understanding of the BMP pathway in relation to GBM pathology. As more genetic information is acquired and as diverse samples are rapidly added to these publically available datasets, we will be able to generate more distinct and conclusive data on the genetic alterations and mutations that are critical regulators in GBM development and progression. In future studies, with the addition of patient data, we will be able to stratify the population based on factors such as tumor subtype, initial or recurrent tumors

or previous treatments to take a more hypothesis driven approach to genomic analysis. Recently GBM genomic studies have begun to explore the extent of intratumoral heterogeneity when stratified by tumor stage or at the single cell level enforcing the need for larger, increased depth of publically available GBM data sets [9,139].

As we begin to combine the rapidly growing knowledge of epigenetic and proteomic information with genomic studies our understanding of GBM tumor biology will vastly increase. Our current understanding of these tumors is clearly not proficient given the dismal, almost uniformly fatal outcome of this disease; however, genomic analyses allow for a future of new diagnostic tests, classifications, treatment combinations and will direct both basic scientists and clinicians towards a future of successful individualized treatments. With the arrival of personalized medicine it is more imperative than ever to gain a further understanding of these heterogeneous tumors at the genetic level for the optimization of new therapies. These studies serve as a starting point informing future research regarding candidate driver mutations, critical genes and pathways to target as we integrate our genomic data and the complex tumor microenvironment.

CHAPTER III: BONE MORPHOGENETIC PROTEIN SIGNALING PROMOTES TUMORIGENESIS IN A MURINE MODEL OF HIGH GRADE GLIOMA

This is a pre-copyedited, author-produced, adapted version of an article accepted for publication in Neuro-Oncology following peer review. The version of record is: Hover, LD., Owens, P., Munden, A., Chambless, L., Hopkins, C., Hong, CC., Moses, HL., & TW. Abel. Bone Morphogenetic Protein Signaling Promotes Tumorigenesis in a Murine Model of High Grade Glioma. Neuro-Onc. In press

INTRODUCTION

High grade gliomas (HGG) are aggressive tumors with a dismal prognosis, despite treatment with surgery, radiation and chemotherapy. Glioblastoma multiforme (GBM), a grade IV astrocytoma, is the most common malignant CNS tumor, with only a 5% five-year survival rate, underscoring our poor understanding of glioma biology and the obvious need for more effective therapies[3].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta (TGFβ) family. During canonical BMP signaling, BMP ligands bind to BMP type I and type II serine-threonine kinase receptor complexes. Upon ligand binding, the type I receptor phosphorylates the regulatory Smads1,5 and 8. These regulatory Smads bind to the co-Smad (Smad4), and the complex is translocated to the nucleus. BMP signaling regulates the transcription of genes affecting critical cell processes, including proliferation, differentiation and apoptosis[39,57]. *Id1-4* gene transcripts are induced in most types of cells by BMP ligands[39]. BMP signaling is tightly regulated by both extracellular antagonists and intracellular modulators such as the inhibitory Smad, Smad6, which acts in a negative feedback manner in response to active BMP signaling[39].

In many types of cancer, BMPs play both tumor promoting and suppressing roles, similar to TGFβ signaling[66,140]. Various lines of evidence suggest that BMP signaling may be important in glioma biology, although contradictory findings appear in the literature[92,93,96]. For example, increased expression of BMP signaling molecules has been associated with HGG[92,93].

Expression of the BMP type IB receptor and the ligand BMP2 were both found to be expressed more frequently and at higher intensity in grade IV gliomas than in low grade gliomas[92,93]. Additionally, BMP type IA receptor has been implicated as a tumor driver in gliomas[90]. Conversely, expression of BMP4 has been associated with low grade gliomas, and positively associated with survival[96]. In addition, several studies have reported that BMP signaling acts as a tumor suppressor on the subpopulation of glioma cells known as glioma stem cells (GSC), by inhibiting proliferation and promoting differentiation[78,79]

Here we present evidence that BMP signaling is present and active in the vast majority of human HGG cells. Furthermore, in a novel transgenic, orthotopic model we show that BMP signaling in transformed astrocytes promotes aggressive tumor behavior via regulation of tumor cell proliferation and migration. Taken together, the findings provide evidence that there are major differences in the role of BMP signaling in the regulation of GSC and more differentiated neoplastic cells.

MATERIALS & METHODS

Transgenic Mice

All animals were housed in the animal care facility at Vanderbilt University and all experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee. All procedures followed the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. *Cre/Kras^{G12D}/p53$^{fl/fl}$* mice were generated and genotyped as described previously [31]. *Cre/Kras^{G12D}/p53$^{fl/fl}$* were bred with conditional *Bmpr1a$^{fl/fl}$* mice [141]. *Cre/Kras^{G12D}/p53$^{fl/fl}$* and *Cre/Kras^{G12D}/p53$^{fl/fl}$/Bmpr1a$^{fl/fl}$* mice were bred to *mT/mG* mice, a double-fluorescent Cre reporter mouse [142]. Mice were bred on a mixed background.

Astrocyte cell culture

Astrocytes were harvested from neonatal (<7 days old) *GFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$/mT^{+}* or *GFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$/Bmpr1a$^{fl/fl}$/mT^{+}* pups as previously described[31]. Astrocytes were harvested from 3 mice per group to establish 3 cell lines per genotype. Astrocytes were grown

as monolayer cultures in T75 cell culture flasks. Recombined cells (GFP-positive, RFP-negative) were isolated using fluorescent activated cell sorting (FACS) with a FACSAria III flow cytometer (BD). Flow cytometry experiments were performed in the VMC Flow Cytometry Shared Resource. DNA was extracted from cultured astrocytes and polymerase chain reaction (PCR) was performed to detect the recombined Bmpr1a allele using the following primers:

5'¶-GGGTAGGTGTTGGGATAGCTG-3'¶

5'¶- TCCGAATTCAGTGACTACAGATGTACAGAG-3'¶.

U87 MG and T98G human glioblastoma cells were obtained from ATCC.

GBM xenograft lines 10, 22 and 46 were obtained from the Mayo Clinic. The cells were maintained by serial transplantation in mice and were characterized as previously described[143].

Orthotopic injections

3-month-old, female, adult C57BL6 mice were purchased from Charles River Laboratories and anesthetized with a ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture. Using a stereotactic frame (Kopf Instruments, Tujunga, CA), 200,000 dissociated astrocytes (resuspended in 2.5 µL sterile PBS) were implanted into the left corpus striatum at a depth of 2.5 mm from the dural surface[31]. The animals were monitored for neurological signs or weight loss for at least 75 days and euthanized if there were signs of significant neurological dysfunction or 20% weight loss.

Histology & Immunohistochemistry

Mice were euthanized, and their brains were fixed, sectioned, and stained with hematoxylin and eosin. Immunohistochemical staining was performed using the following protocol. Five µm sections from paraffin-embedded specimens were mounted on glass slides and dried overnight at 37°C. Sections were heated at 95°C and then deparaffinized in xylene and washed in a graded series of ethanol. Sections were then boiled in Antigen Retrieval Citra Solution, pH 6.0 (BioGenex, Cat#HK086-9K) for 15 minutes at high power and then allowed to simmer for 15 minutes at low power. Endogenous peroxidase activity was quenched in 3% H_2O_2

for 10 minutes. The slides were blocked for non-specific binding using normal goat serum (Vector Laboratories, Cat#S-1000). Slides were incubated with primary antibodies raised against p-SMAD1/5/8 (1:200; Cell Signaling, 9511L), glial fibrillary acidic protein (GFAP) (1:200, Stem Cell Technologies, 01415), GFP (1:1000, Abcam, Cat#ab6556), Nestin (1:1000, EMD Millipore, Cat#MAB353), and OLIG2 (1:500, EMD Millipore, Cat#AB9610). Sections were incubated with primary antibodies overnight at 4°C. After washing the slides with Tris-buffered saline and Tween 20 (TBST), the appropriate secondary antibodies were applied for 60 minutes. Sections were washed and incubated for 30 minutes with VECTASTAIN Elite ABC kit (Vector Laboratories, Cat#PK-6101); DAB peroxidase substrate kit (Vector Laboratories, Cat#SK-4100) was used for color visualization. The sections were counterstained with hematoxylin QS (VECTOR, Cat#H-3404) and coverslipped using Aqua-Poly/Mount, aqueous mounting media (Polysciences, Cat#18606-20).

Microscope and imaging software

Images of the external brain and coronal sections were obtained with a Leica EZ4D dissecting microscope. Images of stained tumor sections were acquired using an Olympus BX21 light microscope with attached Olympus DP70 camera and Olympus cellSens Standard software. Images of live cells were acquired using an Olympus CK40 light microscope.

Tissue Microarray

The tissue-microarray was constructed from archived surgical pathology material derived from tumor resections at Vanderbilt University Medical Center. It was composed of 30 GBMs and 5 grade III gliomas. Two to four cores per specimen were represented, with 14 non-tumor tissue controls. Ages of patients ranged from 18 to 77 years. The TMA was constructed with approval of the Vanderbilt Institutional Review Board, IRB number: 131389. The percentage of positively p-Smad1/5/8 tumor cells within each core was estimated based on the presence of nuclear signal. The intensity of the signal was scored as 0, 1+, 2+, or 3+.

Western Blotting Analysis

Astrocytes or brain tumor tissue were lysed in Roche Complete Lysis-M buffer (Roche, Cat#04719956001). Approximately 35 ug of protein from each sample was used to perform western blots as previously described [31]. Proteins were visualized using a chemiluminescent detection system (PerkinElmer, Cat#NEL122001EA). Primary antibodies were: p-Smad1/5/8 (1:1000, Cell Signaling, Cat#9511L), Smad1 (1:1000, Cell Signaling, Cat#6944S), p-Smad1/5 (1:1000, Cell Signaling, Cat#9516P), GFAP (1:10,000, Stemcell Technologies, Cat#01415), GFP (1:5000, Abcam, Cat#ab6556), and Actin (1:5000, Sigma Aldrich, Cat#A2066). Horseradish peroxidase conjugated secondary antibodies were used: anti-rabbit IgG (1:5000, Thermo Scientific, Cat#31462). Actin levels were determined for each condition to verify that equal amounts of protein were loaded.

Quantitative Real-Time PCR

Astrocytes were seeded at approximately 500,000 cells/well in 6-well plates. Cells were lysed using the RNeasy mini kit (Qiagen, Cat#74106). Complementary DNA was synthesized using the SuperScript Vilo cDNA synthesis kit (Invitrogen, Cat#11754-050). All reactions were performed in triplicate, and each sample was normalized with the threshold cycle of glyceraldehyde-3- phosphate dehydrogenase to obtain the Δ cycle threshold (Ct) value. SYBR Green fluorochrome was used to perform the real-time (RT) PCR reaction. Standard curve was obtained to calculate the Ct values to obtain gene expression values and graphs. Primer sequences are listed in **Table 5**.

Trypan Blue Exclusion

Cell viability was determined using trypan blue exclusion. Approximately 50,000 cells were plated in 200uL supplemented DMEM F:12. Cells were trypsinized and trypan blue stain, 0.4% (Gibco, Cat#15250) was added. Percent viability was determined using the Invitrogen Countess.

MTT assay

Cell proliferation was measured using the MTT Cell Proliferation Assay (ATCC, Cat#30-1010K). Approximately 10,000 cells were plated in a 96-well plate in triplicate in 100uL supplemented DMEM F:12. Cells were treated with DMSO, BMP4 (100ng/uL) or DMH1(3uM-100uM) for 48 hours at 37°C. After 48 hours of incubation, 10uL MTT reagent was added to each well and incubated for 6 hours. 100uL detergent reagent was added to each well, and the 96 well plate was placed in the dark overnight at RT. Approximately 16-18 hours later the absorbance was read at 570nm.

Invasion Assay

25,000 cells were seeded on Matrigel invasion chambers in quadruplicate (BD BioCoat 8.0µm, Cat#354483) for 24 hours. Cells that had migrated to the opposing side of the filter were fixed in 10% buffered formalin overnight and were stained with hematoxylin overnight (Sigma Aldrich, Cat#MHS16). All cells that had migrated per invasion chamber were counted and averaged.

Thymidine Incorporation

Approximately 25,000 cells were plated in a 24-well plate in 500uL supplemented DMEM F:12 media. 24 hours after plating, cells were treated with DMSO or DMH1 (3uM-100uM) for 24 hours. Astrocytes were pulsed with 4µCi of tritiated thymidine per well (PerkinElmer). After 2 hours, the cells were fixed with 1 ml 10% trichloroacetic acid for 30 minutes at room temperature (RT), followed by 2 additional washes with 10% trichloroacetic acid. DNA was solubilized by incubation in 200µl 0.2N NaOH for 30 minutes at RT. Radioactivity was counted using 100 µl of solubilized DNA in 2ml scintillation fluid. Each cell line was plated in quadruplicate.

Scratch Assay

Cells were plated in duplicate at approximately 500,000 cells per well in 6 well-plates. Once cells reached >90% confluency, they were treated with 4ug/ml of Mitomycin-C (Sigma,

Cat#M4287) for 2 hours in normal media. Cells were scratched by marking an “H” in each well. Images of the same area were captured at 0 and 24 hours after the scratch. Quantification of scratch width for each image was measured using Adobe Photoshop CS4. Gap closure was calculated as the width of the gap at 24 hours divided by the original width of the gap.

RESULTS

The BMP signaling pathway is active in the majority of tumor cells in human HGG

Activation of the BMP signaling pathway was assessed by immunohistochemistry for p-Smad1/5/8 using a tissue-microarray composed of 30 GBMs and 5 grade III gliomas. Signal representing p-Smad1/5/8 was present in all samples and was restricted to the cell nucleus. Within each core, an average of 90% of all tumor cells stained positively. However, a range of staining intensity was observed, with tumor cells staining at low, medium or high levels of intensity [**Figure 7**]. The average percentage of tumor cells that stained positively ranged from 71-100% within each tumor. Scores were calculated based on the percentage of cells at each intensity level (0,1,2,or 3). Scores ranged from 13 to 300 [**Figure 8**].

Astrocytes cultured from transgenic *hGFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$/Bmpr1a$^{fl/fl}$* mice show loss of the BMP receptor and impaired response to BMP ligand

We previously established a transgenic, orthotopic transplant model in our laboratory using the human GFAP (*hGFAP*) promoter and Cre/lox technology to simultaneously drive oncogenic *Kras* (*Kras^{G12D}*) expression while deleting *p53* in astrocytes[31]. To test the hypothesis that BMP signaling promotes gliomagenesis, mice harboring floxed BMP type IA receptor (*Bmpr1a)* alleles were used to establish quadragenic, *hGFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$/Bmpr1a$^{fl/fl}$* mice (Fig. 2A). Preliminary studies showed that heterozygous and homozygous knockout of the *Bmpr1a* receptor alone had no detectable effect on brain development. All mice with genetic deletion of *Bmpr1a* showed normal viability and fertility. Both *hGFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$* and

Gene	Forward Primer	Reverse Primer
Bmpr1a	CGCGTGCGAATCAGACAATG	ACCAGTGCCATGGAGCATAC
Id1	TGAAGTCGGGACCACCGGAGG	GGCTGGAACACATGCCGCCT
Smad6	GTGTTGCAACCCCTACCACT	AGGAGGAGACAGCCGAGAAT
Olig1	TCTTCCACCGCATCCCTTCT	CCGAGTAGGGTAGGATAACTTCG
Olig2	GCGGAACCCCGAAAGGTGTG	TTTGAGGTGCTCGCTGCGGA
Prom-1	CCTTGTGGTTCTTACGTTTGTTG	CGTTGACGACATTCTCAAGCTG
Itgb4	GCAGACGAAGTTCCGACAG	GGCCACCTTCAGTTCATGGA
Itgb7	ACCTGAGCTACTCAATGAAGGA	CACCGTTTTGTCCACGAAGG
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Table 5. SYBR qPCR primer sequences

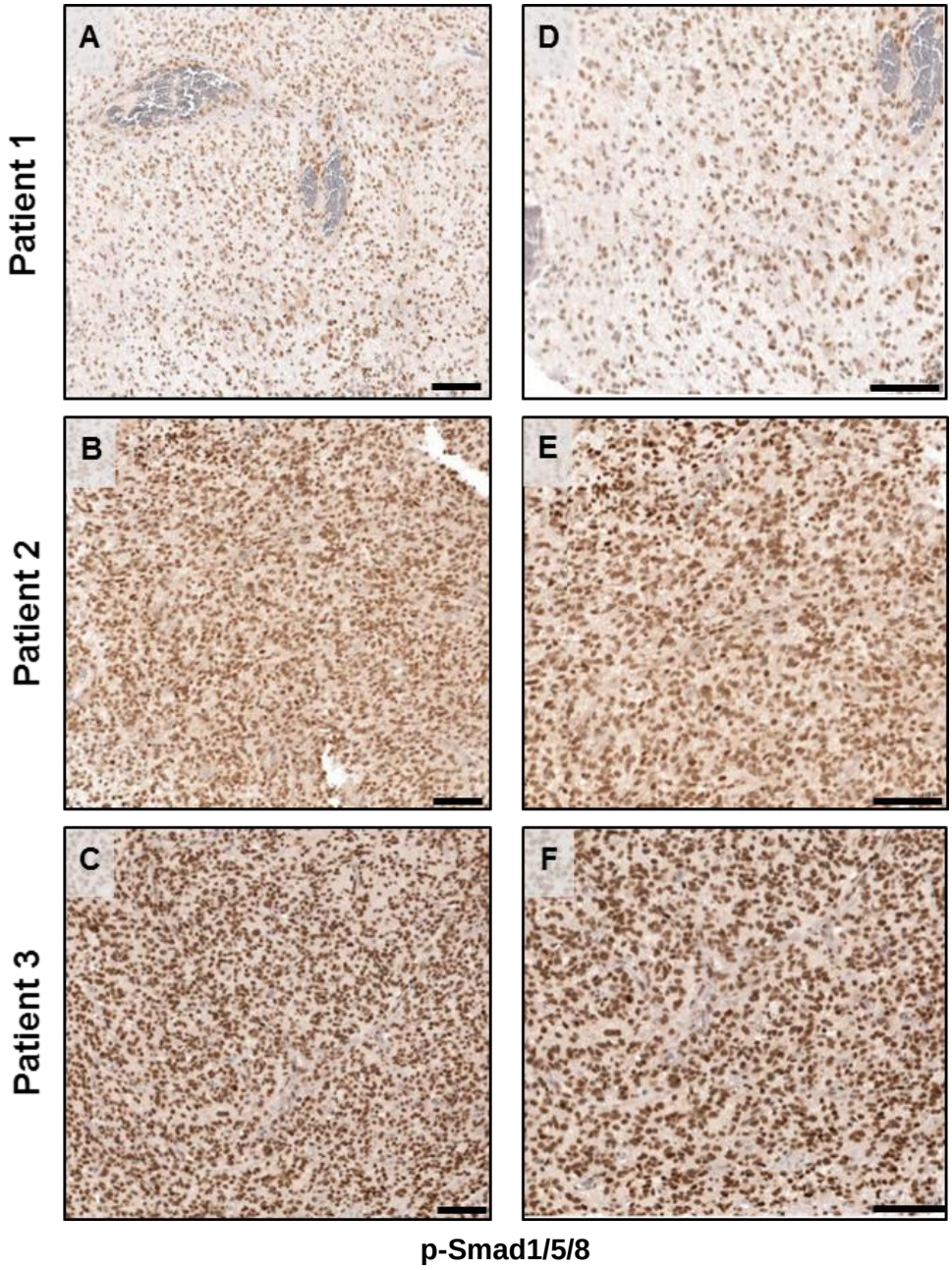


Figure 7. BMP signaling is active in most tumor cells in human HGG

Immunohistochemistry with an antibody against p-Smad1/5/8 was performed on a tissue microarray consisting of 35 samples of human HGG. p-Smad1/5/8 expression was observed in all HGG at varying levels of intensity, in most of the tumor cells. Examples of low (A,D), intermediate (B,E), and high (C,F) intensity staining are shown in tumors from 3 GBM patients at 40x (A-C, scale bar = 100 microns) and 100x (D-F, scale bar =100 microns) magnification

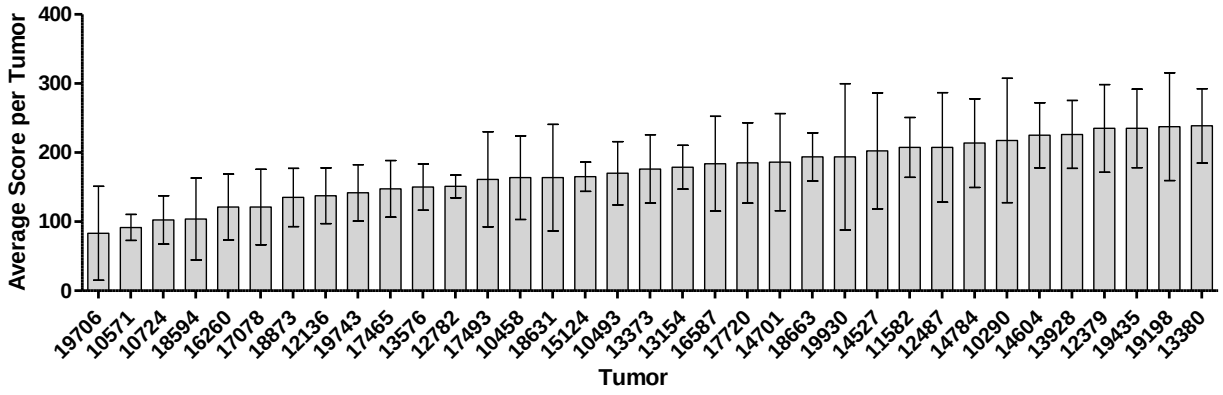


Figure 8. Average score of pSmad1/5/8 staining for all TMA glioma cases

Within each core a total score was determined by estimating the percentage of tumor cells at negative, low, medium or high intensity levels (0-3). The percentage of cells was multiplied by the intensity level (0,1,2,or 3) and added together for a total score per core (0-300). The average score was determined from all cores (2-4) per tumor. Bars indicated standard error of the mean (SEM).

hGFAP-Cre/Kras^{G12D}*/p53*$^{fl/fl}$*/Bmpr1a*$^{fl/fl}$ mice were mated with *mTom*$^{+}$ mice to introduce a Cre reporter gene to monitor recombination.

Previously, we observed that *hGFAP-Cre/Kras*^{G12D}*/p53*$^{fl/fl}$ mice develop neurological symptoms such as seizures or limb paralysis by 4 to 8 weeks of age [31]. In that study, all mice that survived 30-39 days showed the presence of HGG upon histological examination of the brain. Moreover, 9/9 mice examined after one week of age showed focal areas of glioma. However, in the present study, with the additional genetic deletion of *Bmpr1a*, no neurological symptoms were seen in mice living over 200 days. In addition, the brains of two *hGFAP/Cre/Kras*^{G12D}*/p53*$^{fl/fl}$*/Bmpr1a*$^{fl/fl}$ mice were examined approximately 1 month after birth. Histopathologic analysis of these brains showed rare, focal collections of atypical cells, yet there was no evidence of HGG [**Figure 9**]. Previous studies have shown embryonic expression of the *hGFAP* promoter in CNS progenitor cells [144–146]. Therefore, recombination is present at birth and postnatally in multiple cells types, including neurons and glia.

Since our main interest was to determine the role of BMP signaling specifically in the transformed astrocyte population, Astrocytes were harvested from the cortex of neonatal *hGFAP-Cre/Kras*^{G12D}*/p53*$^{fl/fl}$*/mTom* (BMPR1a-intact) *or hGFAP-Cre/Kras*^{G12D}*/p53*$^{fl/fl}$*/Bmpr1a*$^{fl/fl}$*/mTom* (BMPR1a-KO) pups (p<7 postnatal days) and grown under standard conditions as adherent monolayers[147]. Similar culture methods resulted in cultures that were greater than 98% astrocytes[148]. Transformed astrocytes were harvested from 3 mice of each genotype and maintained in culture. Astrocytes of both genotypes grew robustly under these conditions.

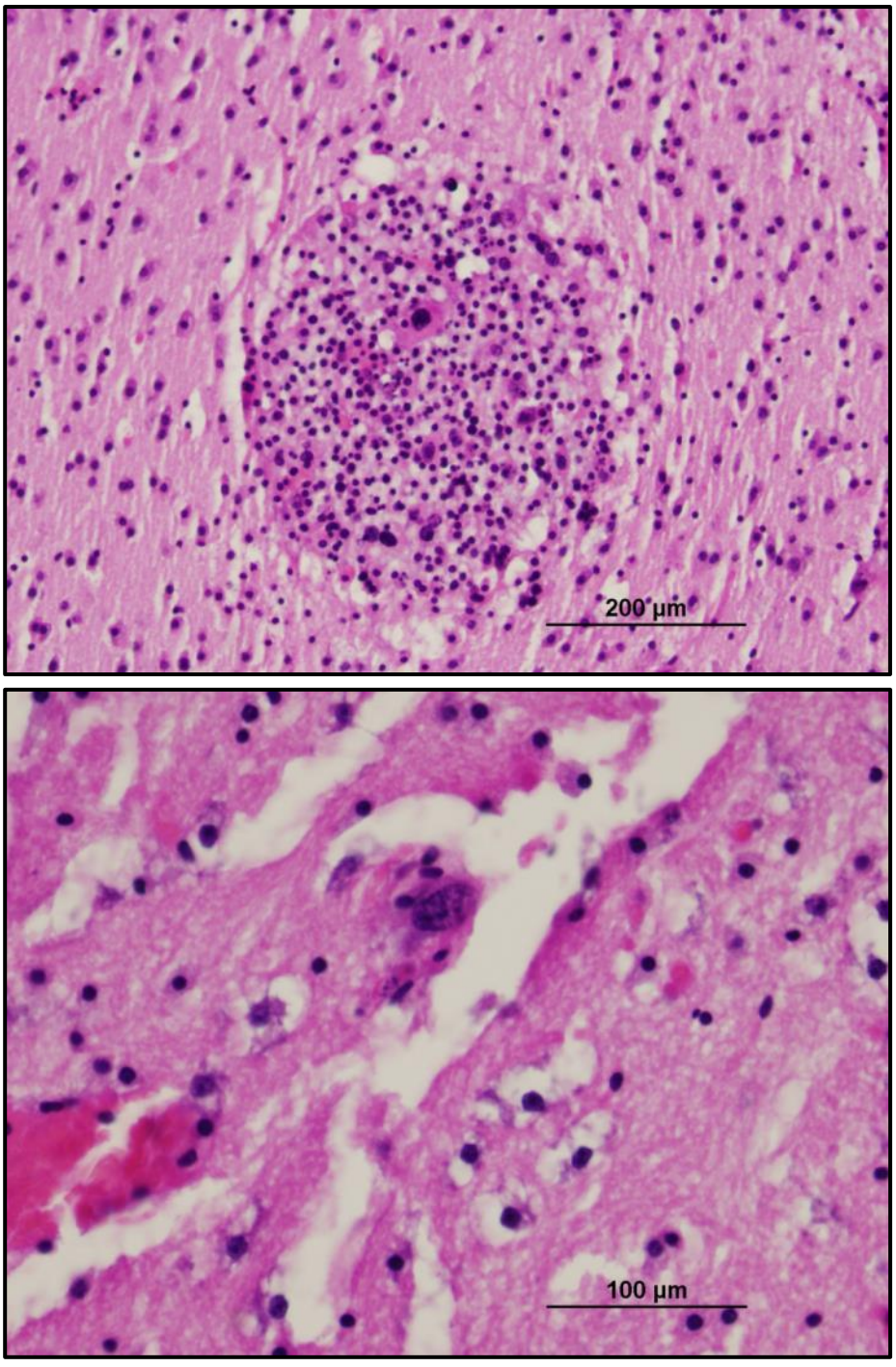


Figure 9. Spontaneous tumor cells and tumor nodules in *hGFAP/Cre/Kras^{G12D}/p53$^{fl/fl}$/Bmpr1a$^{fl/fl}$* mice

Multiple tumor cells and nodules of tumor cells were found in brains from BMRP1a KO mice at approximately 3 weeks of age, however the presence of HGG was not present.

mTom mice constitutively express tdTomato (mT). With Cre activity, mTomato is excised and GFP (mG) is expressed. Thus, monitoring of mGFP expression allows for assessment of Cre activity. The average purity of recombined cells was 73% GFP+ (indicating active Cre driven recombination) in initial astrocyte cultures. Primary astrocyte cultures were subjected to fluorescent activated cell sorting (FACS), gating on the $mGFP^+/RFP^-$ population [**Figure 10A**]. Sorted $mGFP^+RFP^-$ astrocytes were maintained in culture, and subsequent experiments were conducted with pure populations of recombined astrocytes.

Recombination PCR performed on DNA isolated from astrocytes showed recombination of *Bmpr1a* in all BMPR1a-KO astrocyte cell lines [**Figure 10B**]. In addition, quantitative real-time PCR (qPCR) analysis showed the presence of *Bmpr1a* mRNA transcript at varying levels in BMPR1a-intact astrocyte cell lines, while *Bmpr1a* mRNA transcripts were undetectable in BMPR1a-KO astrocyte lines [**Figure 11B**]. Loss of canonical BMP signaling was assessed by examining the mRNA expression for primary BMP downstream targets, *Id1* and *Smad6*. In response to BMP ligand (BMP4) treatment, both *Id1* and *Smad6* expression was significantly lower in BMPR1a-KO astrocytes than in BMPR1a-intact cells [$p<0.001$, $p<0.05$ respectively, **Figure 11C**]. In addition, we analyzed phosphorylation of Smads1/5/8 by western blot. Treatment of BMPR1a-intact astrocytes with BMP4 resulted in robust phosphorylation of Smads1/5/8 [**Figure 11D**]. However, phospho-Smads1/5/8 were undetectable by western blot in BMP4-treated BMPR1a-KO cells, indicating loss of canonical BMP signaling [**Figure 11D**].

Deletion of BMPR1a increases survival in immunocompetent mice with orthotopic implants

Recombined astrocytes were injected into the striata of immunocompetent, adult, host mice (n=10 per group). The control group received transformed BMP-intact astrocytes, while the other group received BMPR1a-KO astrocytes. Tumors formed as a result of both BMPR1a-intact and BMPR1a-KO injections. All tumors that formed were highly invasive with gross

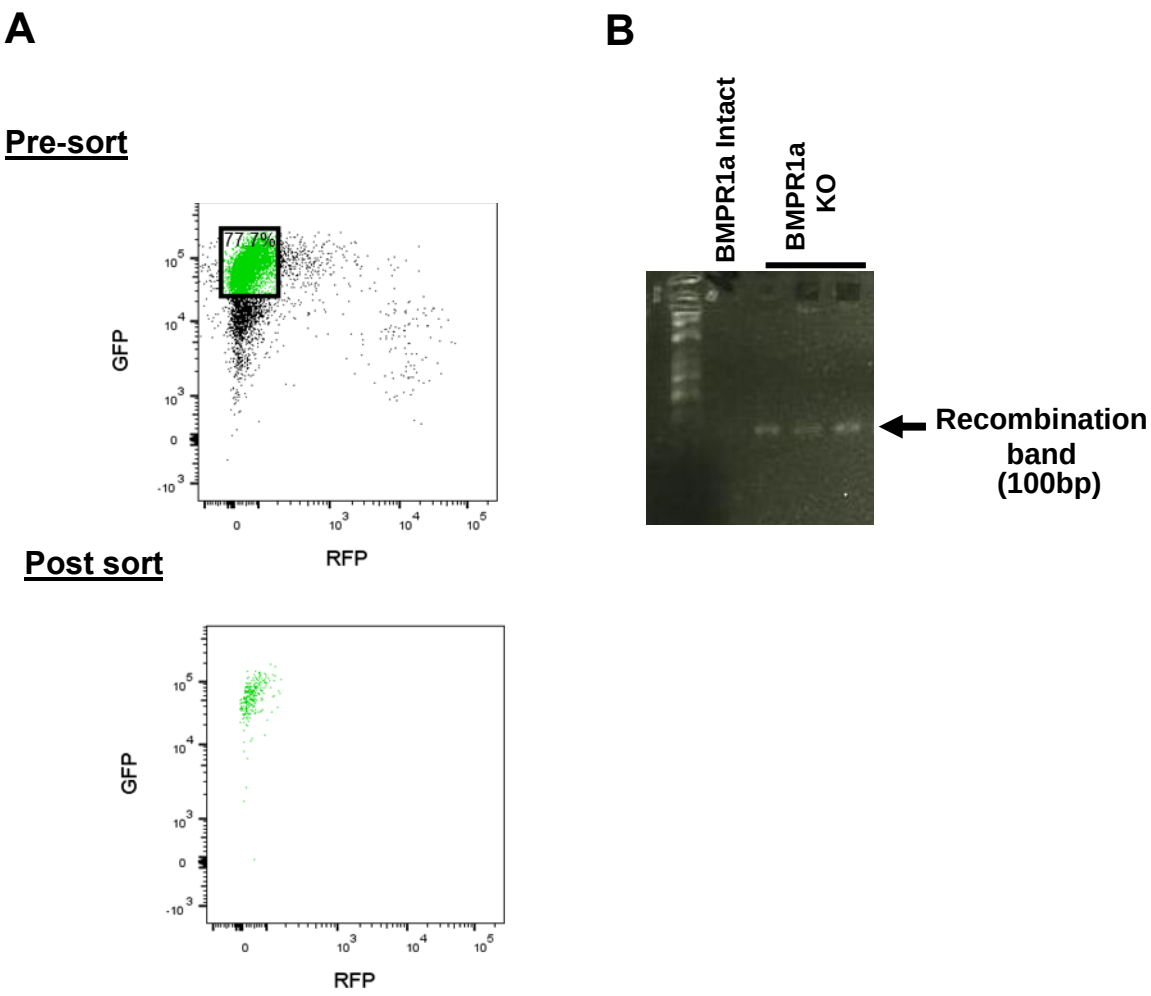


Figure 10. Fluorescence activated cell sorting (FACS) was used to sort initial astrocyte cultures to obtain secondary cultures highly enriched for recombined cells

A) Initial astrocyte cultures were sorted for GFP-positive, RFP-negative cells using (FACS). Sorted cells were re-plated and used for all experiments. B) PCR analysis shows the presence of the recombined *Bmpr1a*$^{fl/fl}$ allele in three BMPR1a-KO cells lines (lanes 2-4) and no recombination for a BMPR1a-intact cell line.

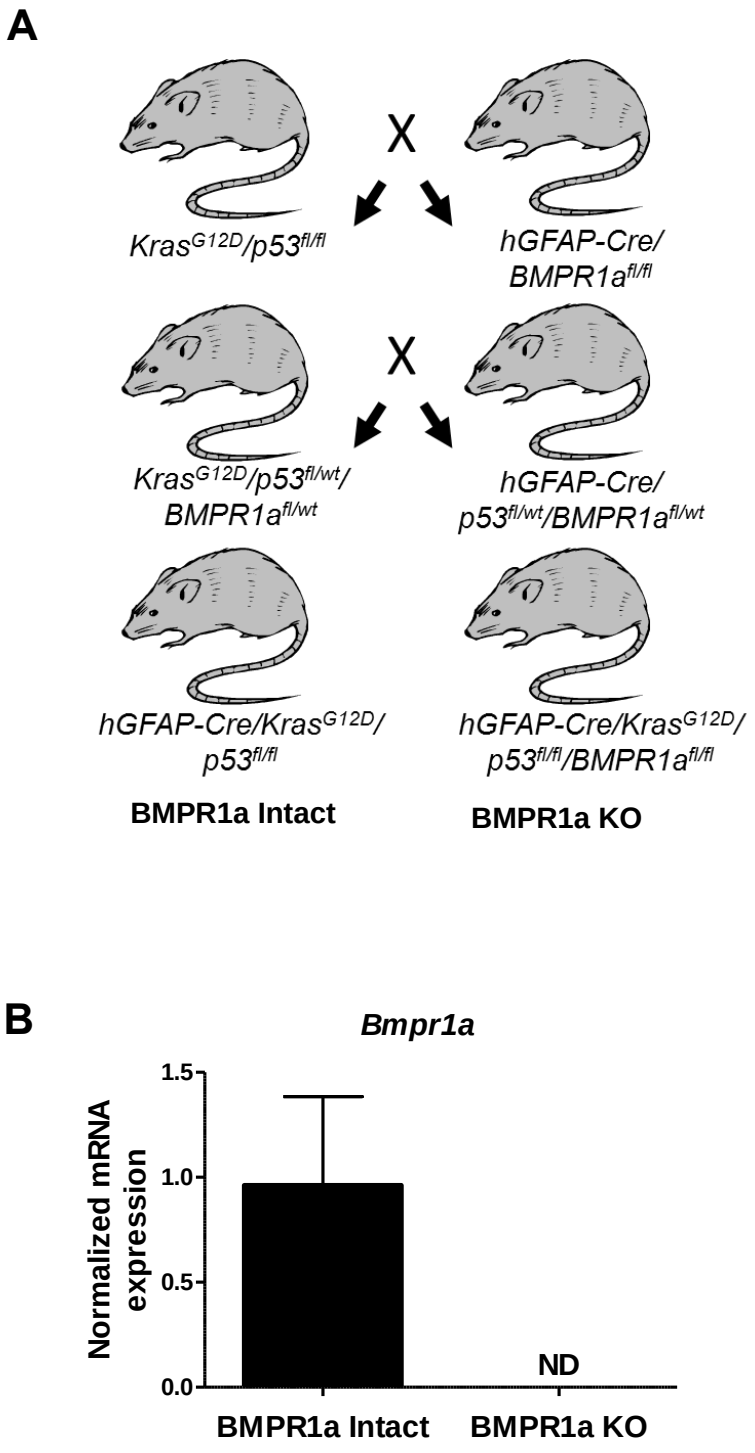


Figure 11. Generation and characterization of transformed astrocytes with genetic loss of BMPR1a

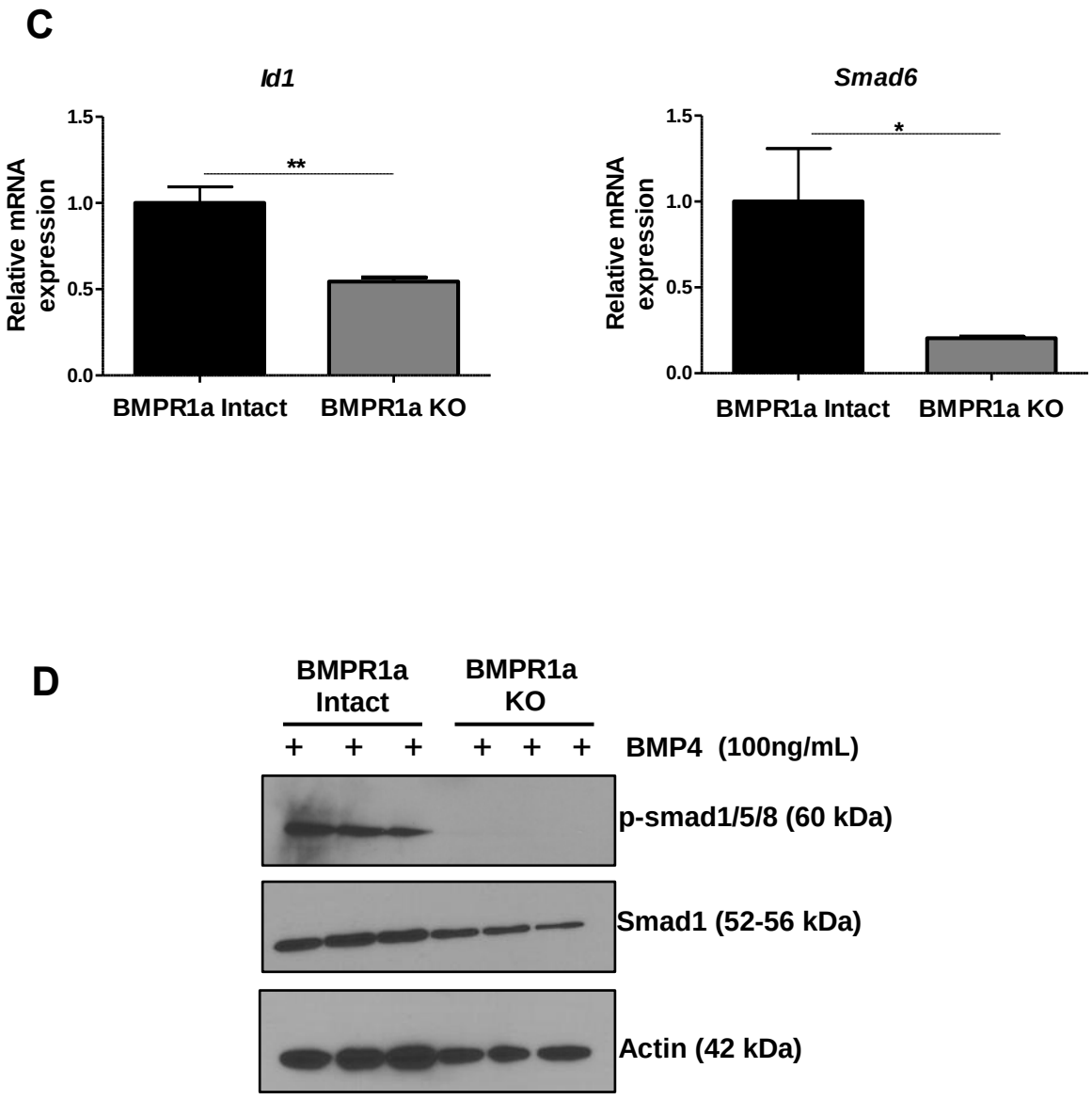


Figure 11. Generation and characterization of transformed astrocytes with genetic loss of BMPR1a

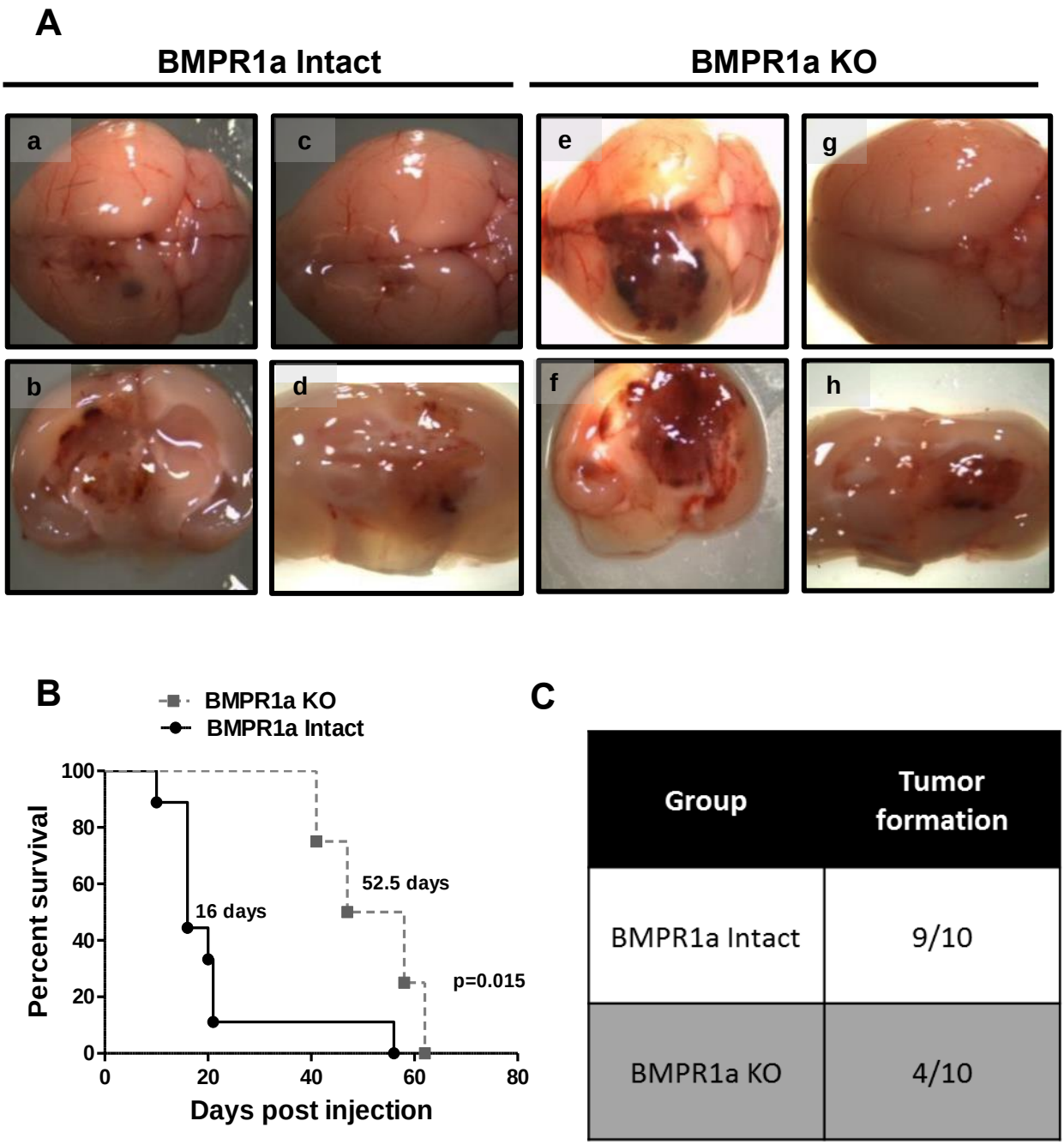
Figure 11. Generation and characterization of transformed astrocytes with genetic loss of BMPR1a

A) Breeding scheme used to generate mice with constitutively active *Kras* (*Kras*^{G12D}), homozygous deletion of *p53* (*p53*$^{fl/fl}$), with and without homozygous deletion of the type IA BMP receptor (*Bmpr1a*$^{fl/fl}$). Mice with oncogenic *Kras* and homozygous deletion of *p53* are termed BMPR1a-intact. Mice with the addition of *Bmpr1a*$^{fl/fl}$ are termed BMPR1a-KO. B-D) Validation of BMPR1a KO. B) mRNA expression of *Bmpr1a* in three BMPR1a-KO transformed astrocyte cell lines was not detected (ND). C) The mRNA expression of the downstream signaling targets of the BMP pathway, *Id1* and *Smad6* were significantly decreased in BMPR1a-KO transformed astrocytes in comparison to BMPR1a-intact transformed astrocytes in response to 24-hour BMP4 treatment (n=3 per group). A two-tailed student's t-test was performed to compare the mean mRNA expression. Bars indicate SEM. *p<0.05, **p<0.01. mRNA is normalized to *Gapdh* levels and relative to BMPR1a-intact

hemorrhage and necrosis. [**Figure 12A**]. However, BMPR1a-intact astrocytes were more effective in forming tumors than BMPR1a-KO astrocytes [9/10 vs 4/10 p=0.06, **Figure 12C**]. In addition, BMPR1a-intact astrocytes formed more aggressive tumors, with a median survival of 16 days compared to 52.5 days in the BMPR1a-KO group [p=0.015, **Figure 12B**].

Histopathologically, the tumors that developed from BMPR1a-intact and BMPR1a-KO astrocytes were similar in appearance [**Figure 13A,a,b,c,d**]. Tumors showed the characteristic features of human HGG, including infiltrating, pleomorphic cells, [arrow **Figure 13A, d**], necrosis, and many mitotic figures [arrow **Figure 13A,c**]. Tumors were highly infiltrative, often invading both cerebral hemispheres as well as the brainstem. Immunohistochemical (IHC) analysis showed GFP expression in tumor cells of both groups of mice, consistent with recombination of the mTomato reporter [**Figure 14A]**.

We examined BMP signaling as measured by phosphorylation of Smads1/5/8. There was diminished p-Smads1/5/8 in BMPR1a-KO tumor cells compared to BMPR1a-intact tumors, indicating decreased BMP signaling in BMPR1a-KO tumor cells [**Figure 13A. e,f**]. We also examined the expression of GFAP an intermediate filament, which is the primary marker for astroglial cells and is universally expressed within human astrocytic tumors. GFAP expression was equally expressed in both types of tumors [**Figure 13A. g,h**]. Western blot analysis from tumor lysates confirmed the IHC findings [**Figure 13B**]. Because BMPs play a crucial role in mediating differentiation of neural and glioma stem cells, we examined the expression of the neural stem cell markers nestin and OLIG2. We observed a subset of cells that were positive for nestin and OLIG2; however there were no apparent differences in expression levels between the BMPR1a-intact and BMPR1a-KO tumors based on immunohistochemistry [**Figure 14B,C**].



Group	Tumor formation
BMPR1a Intact	9/10
BMPR1a KO	4/10

Figure 12. Reduced engraftment and prolonged survival in mice receiving orthotopic injections of BMPR1a-intact versus BMPR1a-KO astrocytes

Figure 12: Reduced engraftment and prolonged survival in mice receiving orthotopic injections of BMPR1a-intact versus BMPR1a-KO astrocytes

A) Mice receiving orthotopic injections of BMPR1a-intact (a-d) or KO (e-h) transformed astrocytes formed tumors (a-h), often appearing on the surface of the brain as hemorrhagic masses (a,e). Coronal sections showed highly infiltrative tumors with multi-focal hemorrhage and diffuse hemorrhage (b,d,f,h). B) Kaplan-Meier curves showing survival of mice injected with BMPR1a-intact (black line) versus BMPR1a-KO (gray line) tumorigenic astrocytes. The median survival for mice injected with BMPR1a-intact cells was 16 days, compared to 52.5 days in mice injected with BMPR1a-KO cells ($p = 0.015$). C) 9/10 of mice injected with BMPR1a-intact cells formed tumors compared to only 4/10 mice injected with BMPR1a-KO cells.

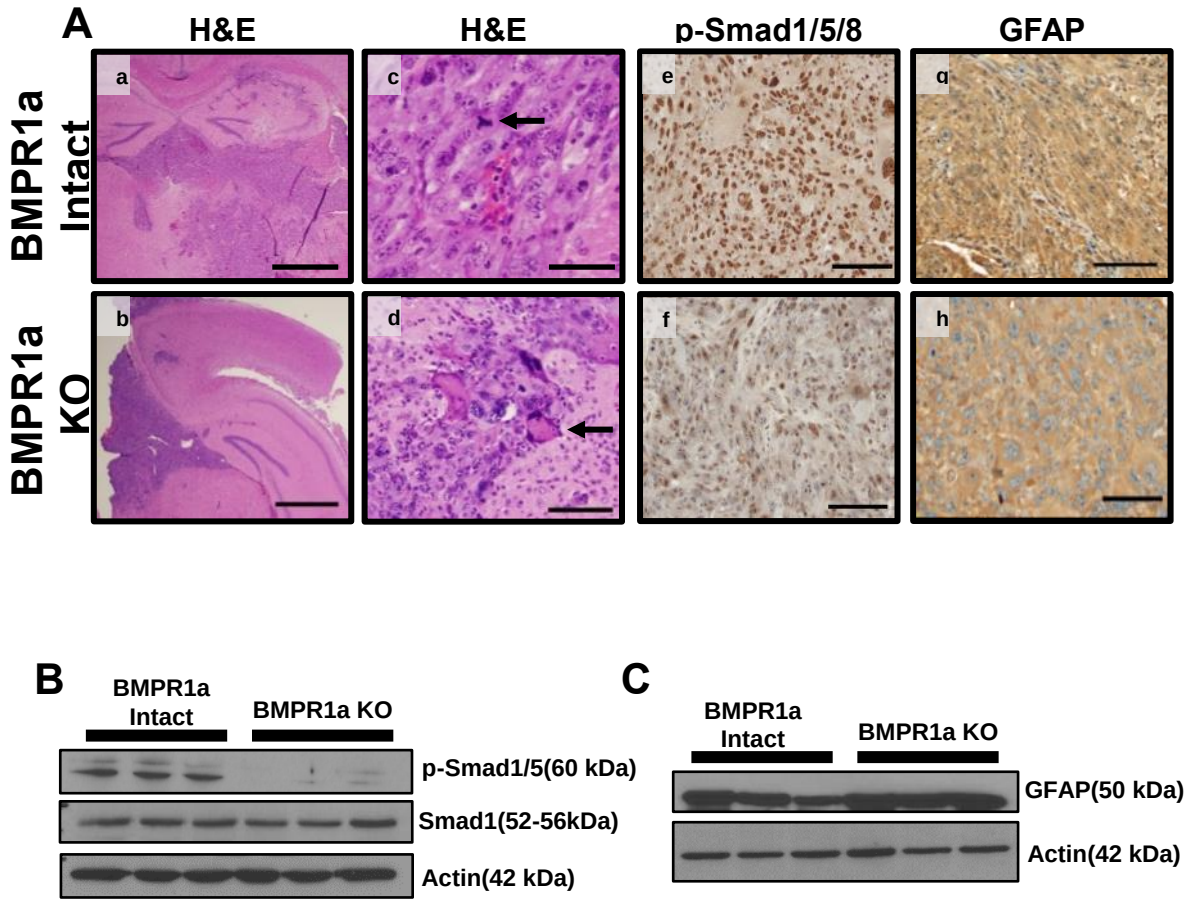


Figure 13. Intracranial tumors derived from BMPR1a-intact and BMPR1a-KO injections show histopathological features characteristic of human HGG

A) Representative H&E-stained sections of BMPR1a-intact (a,c) and BMPR1a-KO tumors (b,d). Tumors are highly infiltrative, with necrosis, mitotic figures (arrow in c), and pleomorphic cells (arrow in d). The histopathology is reminiscent of a human giant cell GBM. BMPR1a-intact tumors show increased p-Smad1/5/8 staining (e) compared to BMPR1a-KO tumors (f) indicating higher levels of BMP signaling (e). GFAP expression is similar in BMPR1a-intact and KO tumors, indicating astrocytic differentiation (g,h). Scale bar 2mm (a,b) Scale bar 200um(c-h) B,C) Western blot analysis on tumor tissue lysates confirms the immunohistochemistry findings.

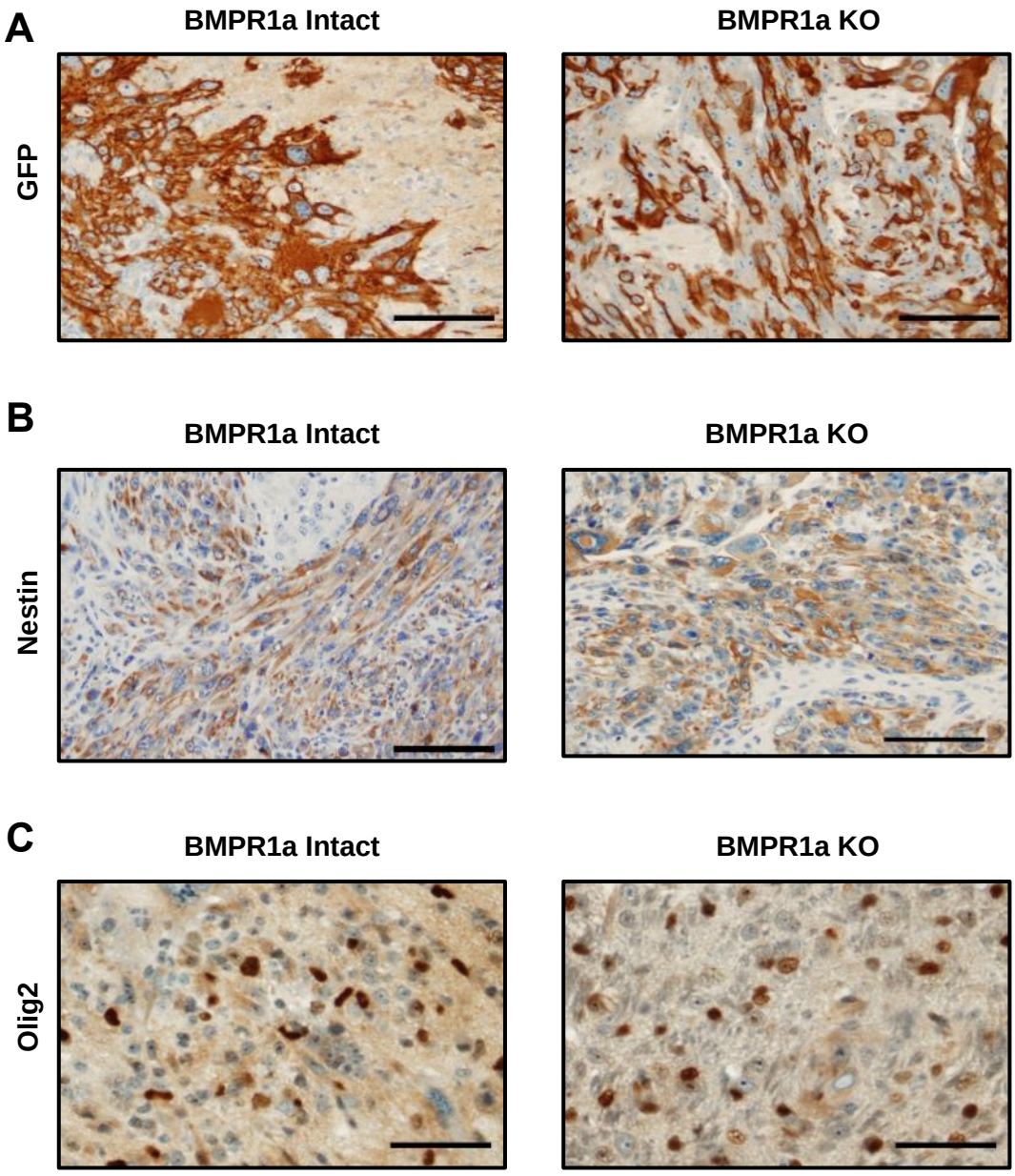


Figure 14. Immunohistochemistry on sections of tumor shows recombination of the mTom reporter construct and focal expression of Nestin and Olig2

A) IHC analysis showed that GFP expression is present in the majority of cells in tumors derived from both BMPR1a-intact and BMPR1a-KO injections B,C) The stemness markers, Nestin (B) and OLIG2 (C) were examined by IHC. Focal, patchy expression was seen for these markers in both BMPR1a-intact and BMPR1a-KO tumors.

BMP signaling promotes proliferation and migration of transformed astrocytes

As loss of BMPR1a markedly increased survival in the orthotopic transplant model, we investigated, *in vitro*, the effect of BMPR1a loss on two hallmarks of cancer: proliferation and invasion. BMPR1a-intact astrocytes proliferated at approximately twice the rate of BMPR1a-KO cells [p = 0.04, **Figure 15A**]. Similar results were obtained with the MTT assay, and cell counts showed no difference in viability between BMPR1a-intact and BMPR1a-KO cells [**Figure 15B,C**].

Next, we compared BMPR1a-intact and BMPR1a-KO astrocytes using a Matrigel invasion assay. Loss of BMPR1a inhibited the ability of transformed astrocytes to migrate and invade, with a two-fold reduction in the number of invading BMPR1a-KO astrocytes compared to BMPR1a-intact astrocytes [p=0.001, **Figure 16**]. In addition, in a scratch assay, the mean wound closure was 73% for BMPR1a-intact cells, compared to a mean of 47% by BMPR1a-KO cells [p=0.002, **Figure 16B,C**]. In parallel, qPCR was performed for a panel of genes known to be involved in migration. Loss of BMPR1a in transformed astrocytes resulted in diminished expression of mRNA for the integrin beta subunits 4 and 7 [**Figure 16D**].

Loss of BMP signaling increases mRNA expression for stemness markers in transformed astrocytes

BMP signaling is known to regulate neural and glioma stem and progenitor cell differentiation. Therefore, we analyzed transcripts for several established neural stem cell markers. Expression levels of *Prom-1* (CD133), *Olig1* and *Olig2* mRNA were significantly greater in BMPR1a-KO astrocytes. *Olig1* and *Olig2* gene expression were increased

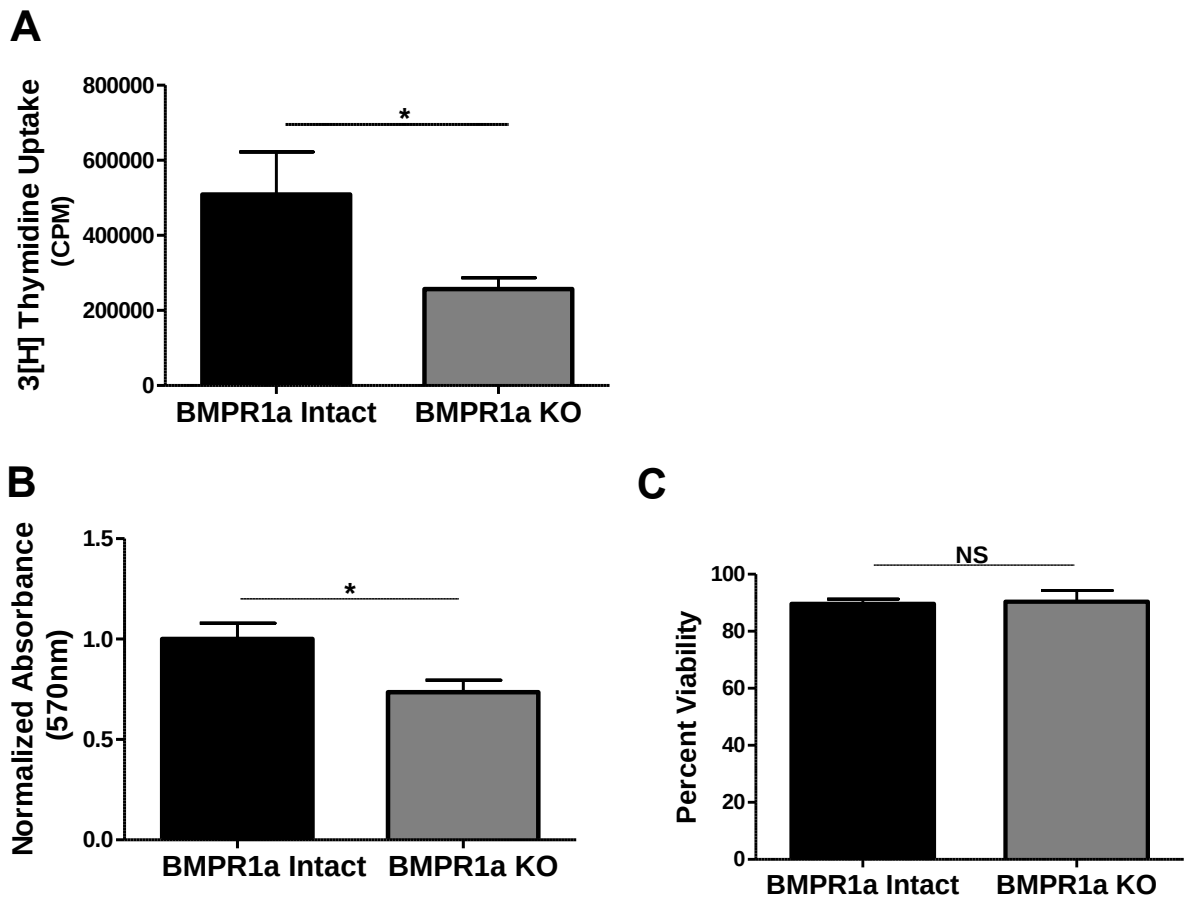


Figure 15. Abrogation of BMP signaling in transformed murine astrocytes inhibits proliferation

A) Tritiated thymidine incorporation assay shows that proliferation is inhibited in BMPR1a-KO astrocytes (gray bars) compared to BMPR1a-intact cells (black bars). Results are expressed as mean counts per minute (CPM) for quadruplicate samples at each time point for 3 cell lines per group. B) MTT analysis shows fewer viable BMPR1a-KO astrocytes after 48 hours of growth compared to BMPR1a-intact astrocytes. Results are expressed as mean absorbance at 570nm for quadruplicate technical replicates with 3 cell lines per group. Absorbance is relative to absorbance for BMPR1a-intact astrocytes. C) Percent viability was equal in both astrocyte populations as measured by live cell counts using trypan blue exclusion

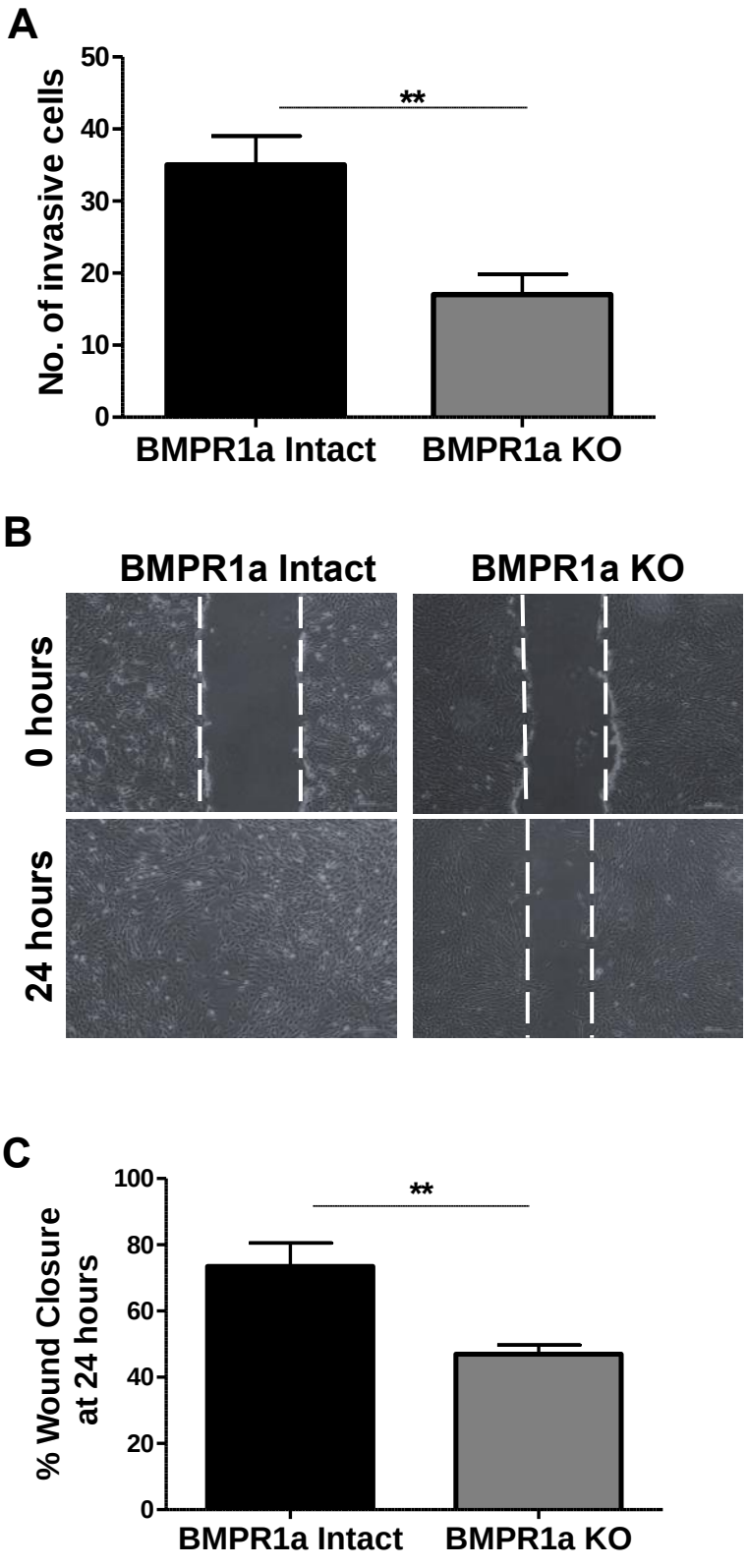


Figure 16. Abrogation of BMP signaling in transformed astrocytes inhibits migration and invasion

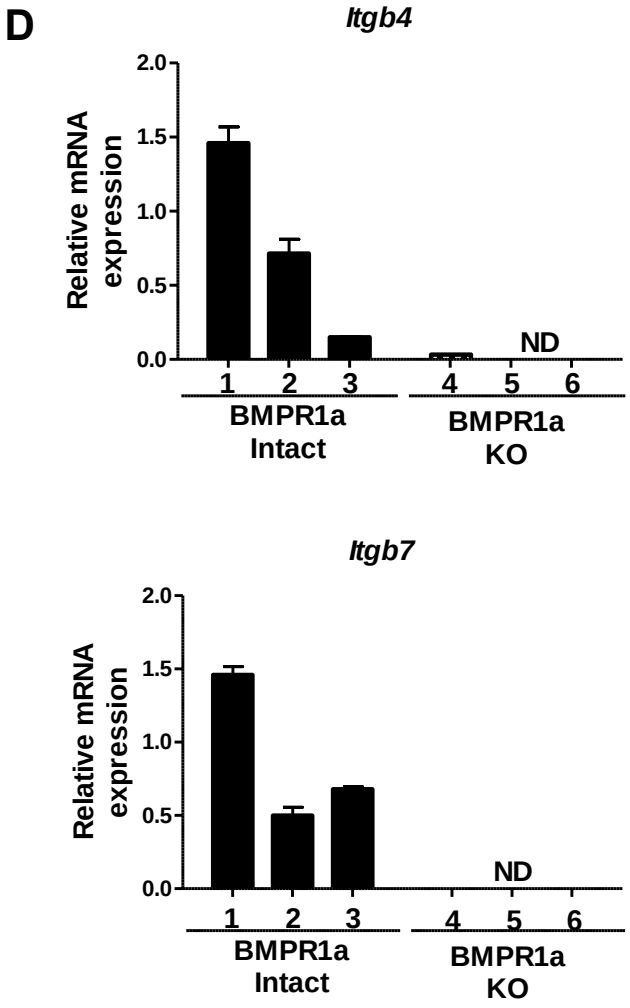


Figure 16. Abrogation of BMP signaling in transformed astrocytes inhibits migration and invasion

Figure 16. Abrogation of BMP signaling in transformed astrocytes inhibits migration and invasion

A) In a 24-hour, Matrigel transwell invasion assay, BMPR1a deletion results in a 50% reduction in the number of cells able to invade. Results are expressed as the mean number of cells able to invade with quadruplicate transwells and 3 cell lines per group. B,C) Scratch assay was performed on coverslips with >90% confluent BMPR1a-intact and KO astrocytes. B) Representative images of BMPR1a-intact and BMPR1a-KO astrocytes at time of the original scratch (0 hours) and at 24 hours (4x).The BMPR1a-intact cells migrated into the wound, resulting in a smaller gap at 24 hours compared to the BMPR1a-KO cells. All cells were treated with Mitomycin-C (MMC) 2 hours prior to performing the scratch assay. D) Integrin beta 4 and 7 mRNA expression levels are decreased or not detected (ND) in BMPR1a-KO astrocytes as measured by qPCR. Numbers indicate separate cell lines. mRNA is normalized to *Gapdh* levels *$p<0.05$ Bars indicate SEM.

approximately 8- and 23-fold respectively [**Figure 17A,B**]. *Prom-1* mRNA levels were 4-fold higher in BMPR1a-KO cells [**Figure 17C**].

DMH1, a small molecule inhibitor of BMP signaling, inhibits astrocytic proliferation and migration *in vitro*

To complement the genetic approach to inhibition of BMP signaling, cells were treated with Dorsomorphin homologue 1 (DMH1), a highly selective BMP type I receptor small molecule inhibitor[149]. BMPR1a-intact, transformed murine astrocytes and a human glioma cell line (U87) were treated with either vehicle (DMSO) or DMH1 for 24 hours. In all cell lines, DMH1 treatment (10μm) decreased the BMP ligand-induced expression of BMP-target genes *Id1* and *Smad6* [**Figure 18.**]. In addition, DMH1, in a dose-dependent manner, inhibited proliferation in tumorigenic murine cell lines and two human GBM cell lines as measured by MTT assay [**Figure 19A,C,E**]. To ensure that these effects were due to decreased proliferation and not drug toxicity, we confirmed the decrease in proliferation by ^{3}H-thymidine incorporation in the 3 transformed murine astrocyte lines [**Figure 19B**]. No effects on cell viability were observed in U87 cells treated with DMH1 [**Figure 19D**]. As DMH1 inhibits all BMP type I receptors, we also observed decreased proliferation of BMPR1a-KO astrocytes upon DMH1 treatment as measured by MTT assay [**Figure 20A**]. However, we treated 3 human GBM cell lines grown as neurospheres with varying concentrations of DMH1and saw no effect on proliferation [**Figure 20B**].

Finally, the effect of DMH1 on cell migration was tested in a scratch assay. DMH1 treatment significantly decreased the migratory ability of BMPR1a-intact cells compared to untreated controls. We observed an average of 30% closure in DMH1 treated cells compared to a mean wound closure of 79% in untreated controls [$p<0.0001$,**Figure 21**].

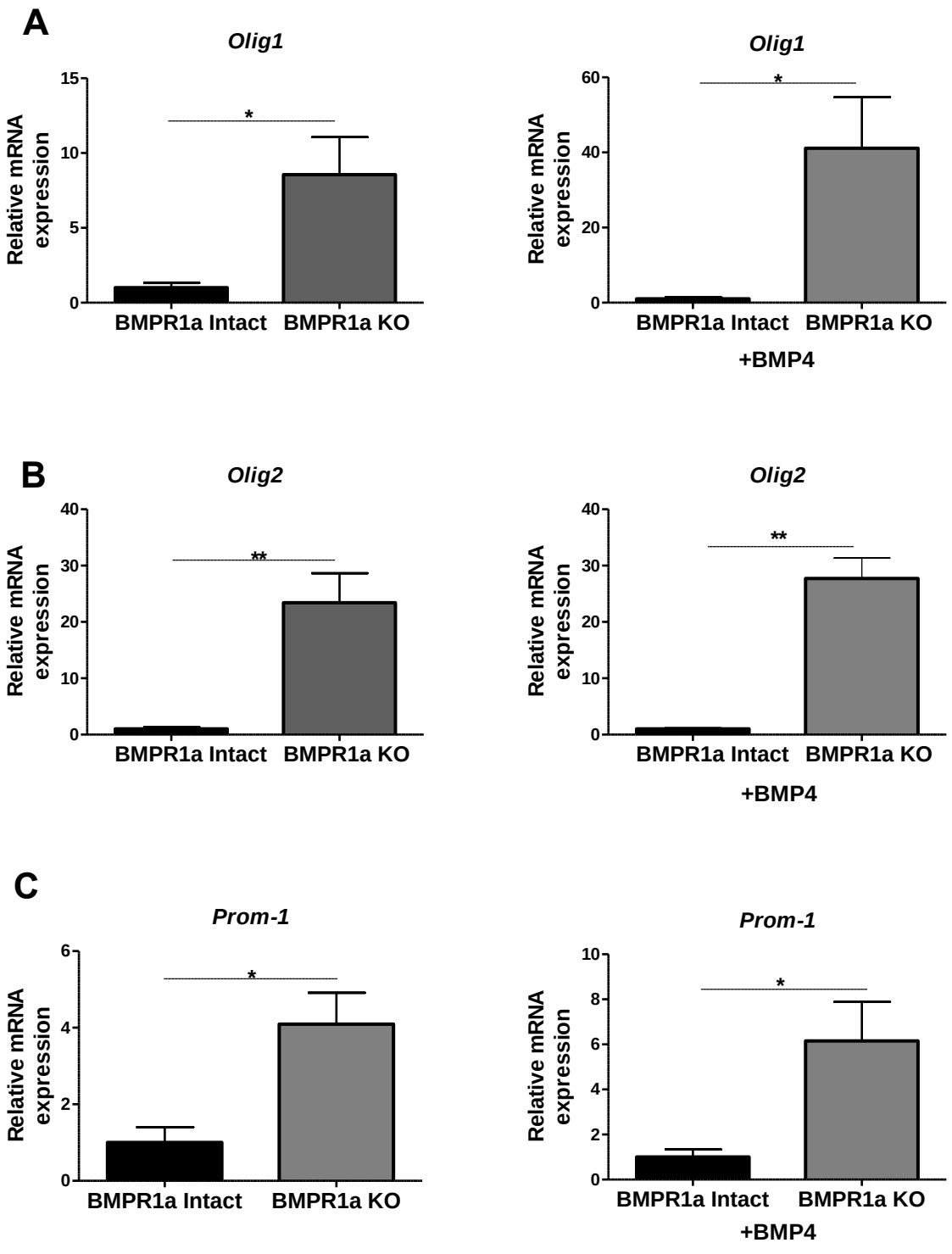


Figure 17. BMPR1a KO, transformed astrocytes show increased mRNA expression of stemness markers

Figure 17 BMPR1a KO, transformed astrocytes show increased mRNA expression of stemness markers

The mRNA expression levels of Olig-1 (A), Olig-2(B) and Prom-1 (C) were higher in BMPR1a KO astrocytes compared to BMPR1a intact astrocytes (n=3 cell lines per group). Treatment with BMP4 (100ng/mL) resulted in a greater fold difference between the intact and KO astrocytes, compared to knockout of the receptor alone (A,B,C). A two-tailed student's t-test was performed to compare the mean mRNA expression. mRNA is normalized to Gapdh levels and relative to BMPR1a intact expression. Bars indicate SEM. *$p<0.05$, **$p<0.01$.

DISCUSSION

BMP signaling is critical for neural development and the regulation of neural progenitor cells[57]. Several lines of evidence suggest an important role for this pathway in gliomagenesis as well, although the details are poorly understood. Previous studies indicate that BMP receptors are present on human glioma cells, and that BMP receptor quantity correlates with tumor grade[92]. The majority of studies regarding BMP signaling in gliomas focus on human GSC in orthotopic transplant models, implicating BMP signaling in the differentiation of GSC and hence as a tumor suppressor in this paradigm[78,79]. However, like other members of the TGF-β superfamily, pro- or anti-tumorigenic effects of BMP may depend on the cellular context in which the pathway is active[66]. Here, we provide evidence that BMP pathway activity extends beyond the GSC compartment, and that BMP signaling fosters tumorigenesis in neoplastic astrocytes through promotion of proliferation and invasion. These data suggest that BMPs may differentially regulate the GSC and "bulk tumor" compartments in HGG.

To assess active BMP signaling in human HGG tissue, we used immunohistochemistry for p-Smad1/5/8 on a series of HGG. Our results showed the presence of nuclear phospho-Smads1/5/8, at varying levels of intensity, in all tumors. Although others have shown the presence of BMP pathway signaling components, including BMP ligands and receptors, the presence of these components is not directly related to pathway activity [92,93,96]. Our data suggest that active BMP signaling is present in the majority of human HGG.

In addition, our data show that BMP signaling is active in about 90% of the tumor cells within a given tumor. The proportion of glioma cells with stem cell-like properties is estimated to range from <1-30%[26,33]. Therefore, it follows that many of the phospho-Smad1/5/8-expressing cells in the tumor samples we analyzed reside in the non-GSC or bulk tumor compartment. These data underscore the importance of understanding the regulation by BMP of glioma biology in the more differentiated glioma compartment.

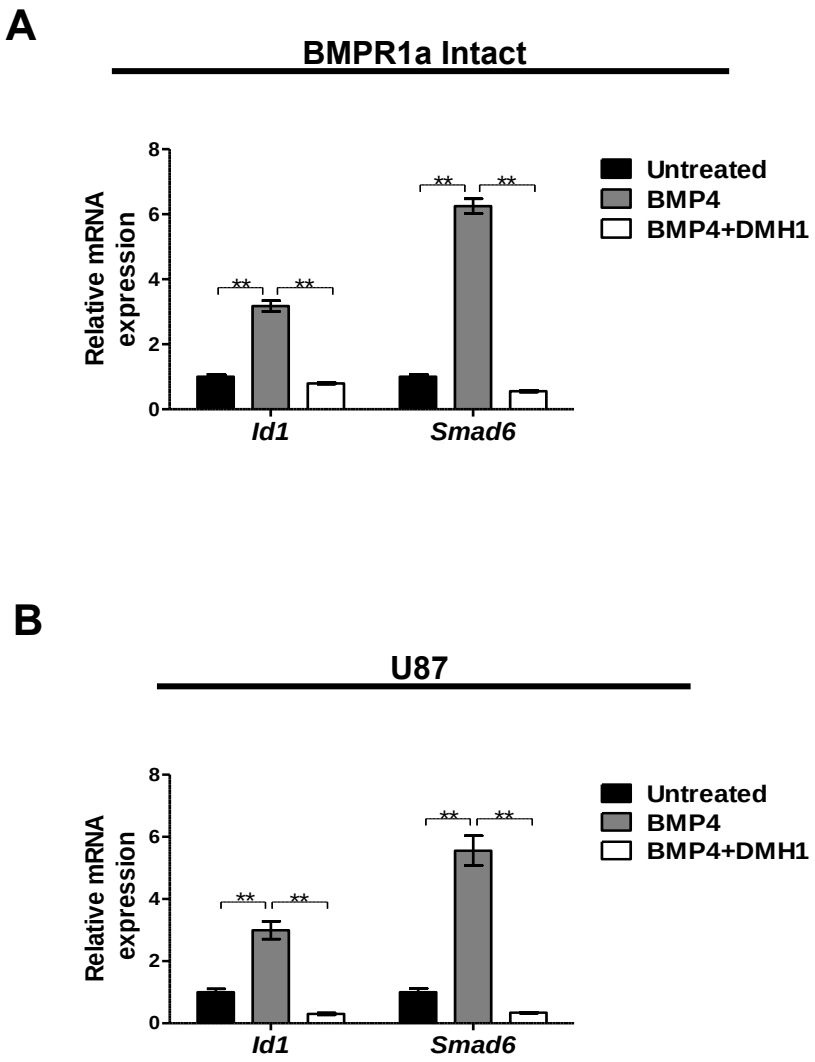


Figure 18. Pharmacological inhibition of BMP decreases BMP induced canonical targets in oncogenic astrocytes

A,B) DMH1 treatment (10μM) decreases BMP-induced *Id1* and *Smad6* expression in murine BMPR1a-intact transformed astrocytes and human GBM astrocytes (U87) as measured by qPCR. A two-tailed student's t-test was performed to compare the mean mRNA expression. mRNA is normalized to *Gapdh* levels and relative to untreated expression. *p<0.05, **p<0.01

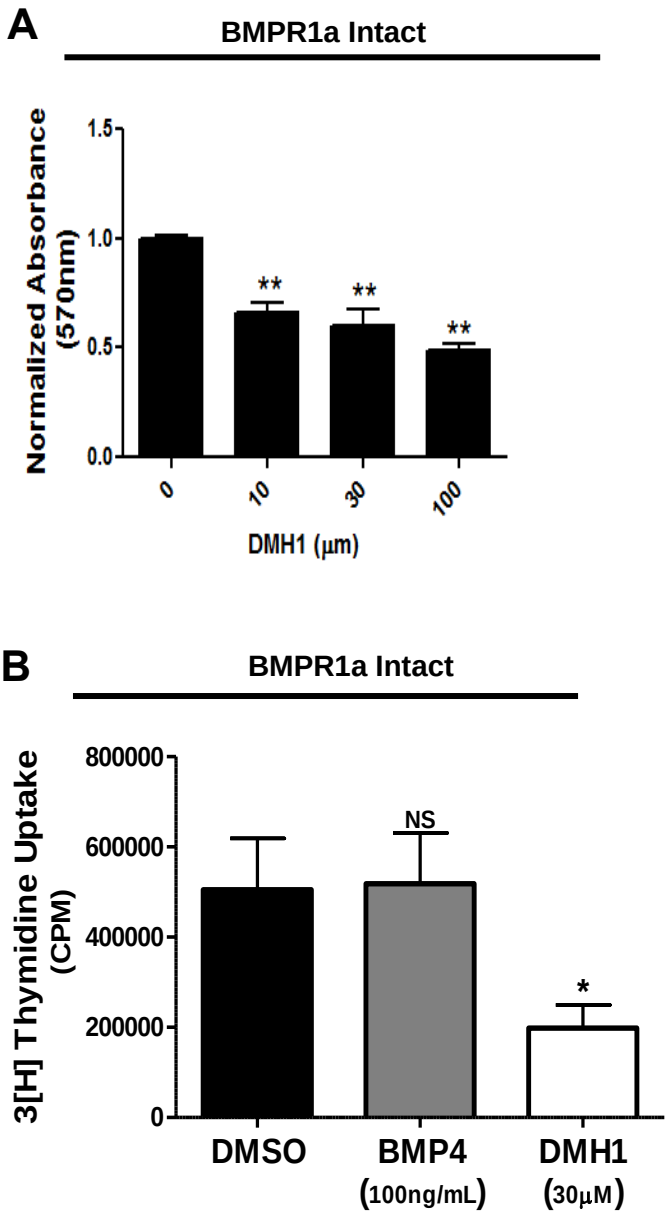


Figure 19. Pharmacological inhibition of BMP decreases proliferation of oncogenic astrocytes

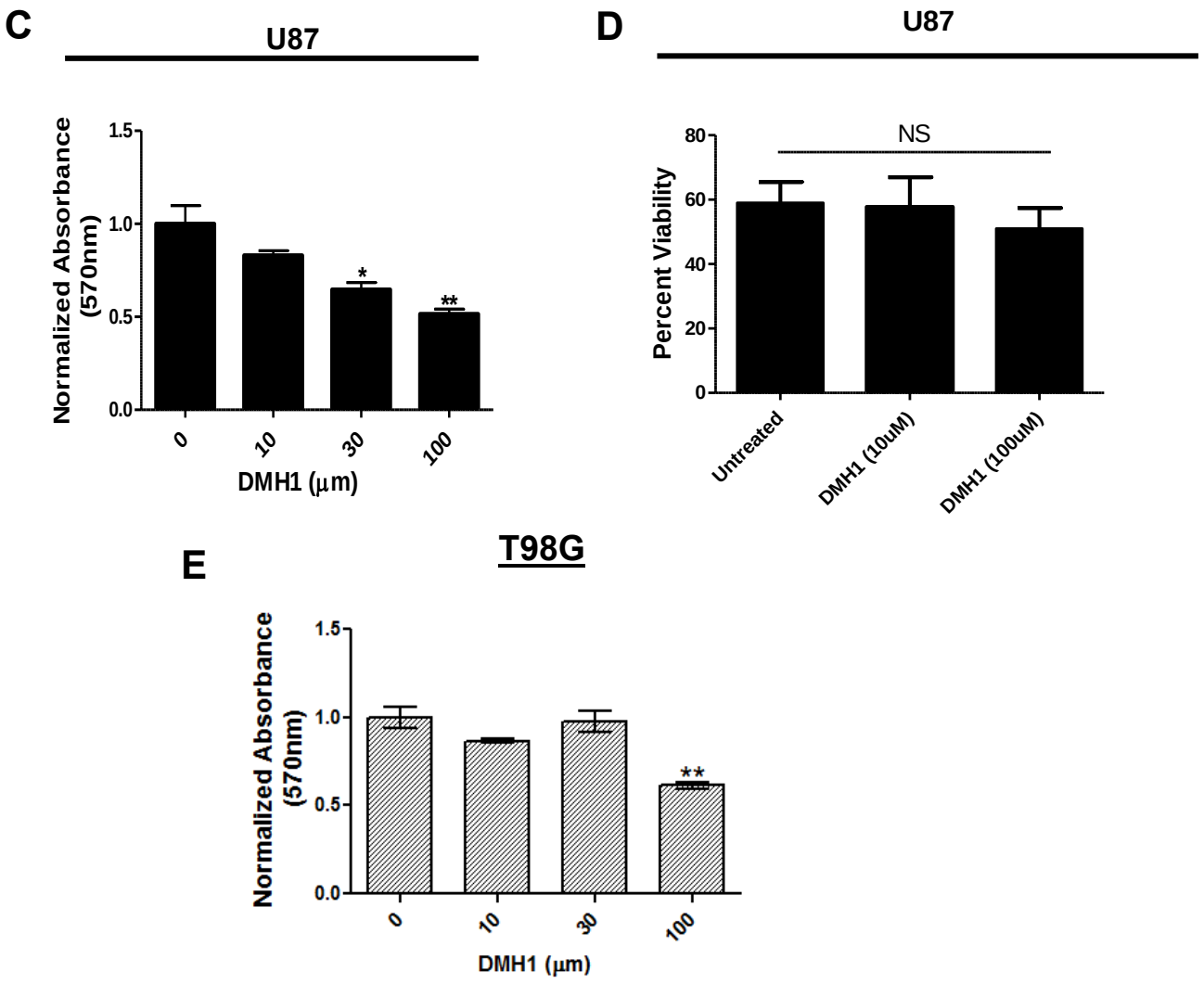


Figure 19. Pharmacological inhibition of BMP decreases proliferation of oncogenic astrocytes

A,C,E) Transformed astrocytes were treated with various concentrations of DMH1. DMH1 inhibited proliferation, in a dose-dependent manner, in both transformed, murine astrocytes and human GBM cells. Significance was determined by ANOVA followed by post-hoc t-tests. B) Treatment with DMH1 (30µM) decreased proliferation in transformed, murine astrocytes as measured by tritiated thymidine incorporation. Results are expressed as mean counts per minute (CPM) for quadruplicate samples for 3 cell lines. D) DMH1 treatment did not affect the viability of U87 cells at 10 or 100µM as measured by live cell counts using trypan blue exclusion. Significance was determined by ANOVA followed by post-hoc t-tests. *p<0.05, **p<0.01

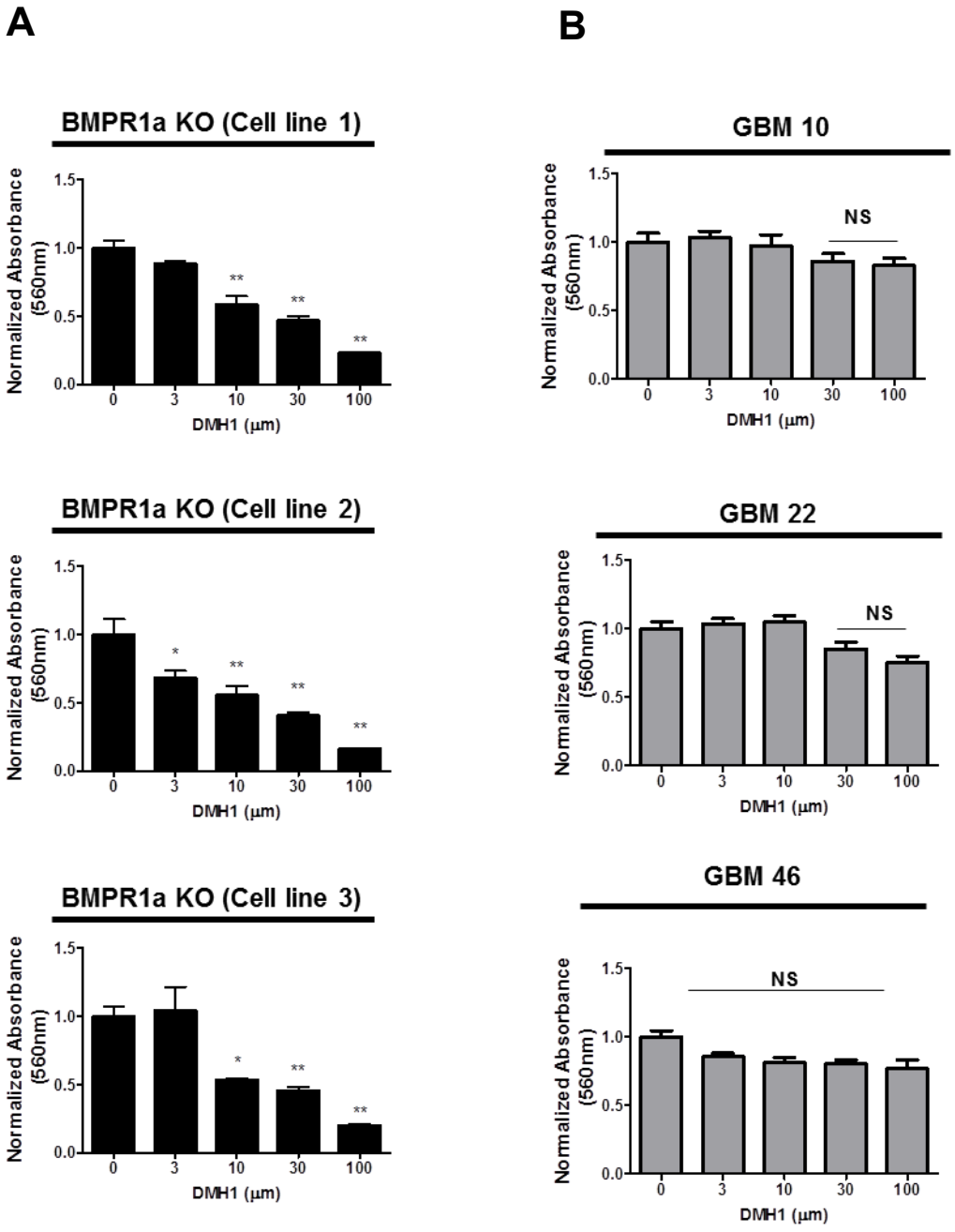


Figure 20. DMH1 decreases proliferation of human GBM cells and Bmpr1a-KO astrocytes, but does not affect the proliferation of human GBM cells grown as neurospheres

Figure 20. DMH1 decreases proliferation of human GBM cells and Bmpr1a-KO astrocytes, but does not affect the proliferation of human GBM cells grown as neurospheres

A) 3 BMPR1a-KO transformed astrocyte cell lines were treated with various concentrations of DMH1 from 0 to 100μm. DMH1 significantly inhibited proliferation in a dose dependent manner. Significance was determined by ANOVA followed by post-hoc t-tests. B) 3 patient GBM xenograft lines grown as neurospheres were treated with various concentrations of DMH1 from 0 to 100μm. DMH1 had no effect on the proliferation at any concentration. *$p<0.05$ **$p<0.01$ NS=Not significant. Bars indicate SEM.

To address the functional role of BMP signaling in tumorigenic astrocytes, we used a transgenic model highly relevant to human HGG. While KRAS mutations generally are not present in human GBMs, Ras pathway activation, by several mechanisms, including copy number gains, and/or mutation and upregulation of upstream receptor tyrosine kinases, is known to occur in over 80% of human GBMs[12,150]. In addition, alterations in cell cycle regulation are common, with p53 dysregulations in up to 87% of human GBMs[12]. Using various methodologies for activating Ras and interfering with cell cycle regulation in neural cells, we and others have developed transgenic murine models of glioma that faithfully recapitulate key clinical and histopathological features of human glioma, allowing us to study BMP signaling in a highly relevant HGG model system[31,145,151,152].

Previously, we showed that when astrocytes from *hGFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$* mice are harvested, maintained in short-term culture, and injected orthotopically in immunocompetent mice fatal HGG form. In the present study, we incorporated BMPR1a$^{fl/fl}$ transgenic mice into a breeding strategy to generate quadragenic *hGFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$/BMPR1a$^{fl/fl}$* mice (BMPR1a-KO mice). We then harvested astrocytes from these animals for further experiments, comparing them to transgenic *hGFAP-Cre/Kras^{G12D}/p53$^{fl/fl}$/BMPR1a$^{wt/wt}$* (BMPR1a-intact) cells. We targeted BMPR1a specifically as it is a critical and necessary receptor during CNS development whereas other BMP type I receptors, such as BMPR1b, are not[153]. In addition, BMPR1a has been shown to promote tumor growth in multiple systems including gliomas[77,90]

As expected, BMPR1a-intact astrocytes formed aggressive gliomas when injected orthotopically in immunocompetent hosts. In contrast, BMPR1a-KO astrocytes engrafted at a lower rate, and survival was prolonged in the host mice. The median survival for mice receiving BMPR1a-KO astrocytes was 52.5 days, more than a 3-fold increase compared to the mice that received BMPR1a-intact astrocytes. The data strongly suggest that BMP signaling via the BMPR1a receptor promotes tumorigenesis in transformed astrocytes.

To investigate the functional role of BMPR1a in transformed astrocytes, we conducted a series of *in vitro* experiments with transgenic astrocytes and two human GBM cell lines. Both genetic deletion of BMPR1a and pharmacologic inhibition of BMP signaling with DMH1 inhibited the proliferation of transformed murine astrocytes *in vitro*. In addition, DMH1 inhibited proliferation in U87 and T98G cells. Because DMH1 inhibits all BMP type I receptors, we observed its effects on BMPR1A-KO cells and found that inhibition of all BMP type I receptors further suppresses proliferation.

Similarly, both genetic deletion of BMPR1a and DMH1 treatment impaired the ability of transformed astrocytes to migrate and invade. Taken together, using both genetic and pharmacological inhibition of BMP signaling in mouse and human cells, our findings suggest that BMP signaling regulates three elements of tumor cell behavior that are essential components in astrocytoma formation and progression: proliferation, invasion and migration. Similar results were recently published in which reduction of BMPR1a by microRNA-656 resulted in decreased tumor growth, proliferation and migration[90].

HGG are characterized by a high proliferation index, and a rapidly growing tumor mass[5]. Our data strongly suggest that BMP signaling promotes proliferation in tumorigenic astrocytes, including U87 cells. This contrasts with previous work with GSC, in which BMP signaling decreased proliferation and enhanced differentiation in this population of tumor cells[78,79]. Our findings are consistent, however, with studies in lung and breast cancer, in which BMPs promote proliferation, while inhibition of BMP signaling reduces cell proliferation[76,154]. Moreover, these findings are in line with published data showing differential effects, depending on developmental context, of signaling through pathways that are implicated in development and cancer[31,140].

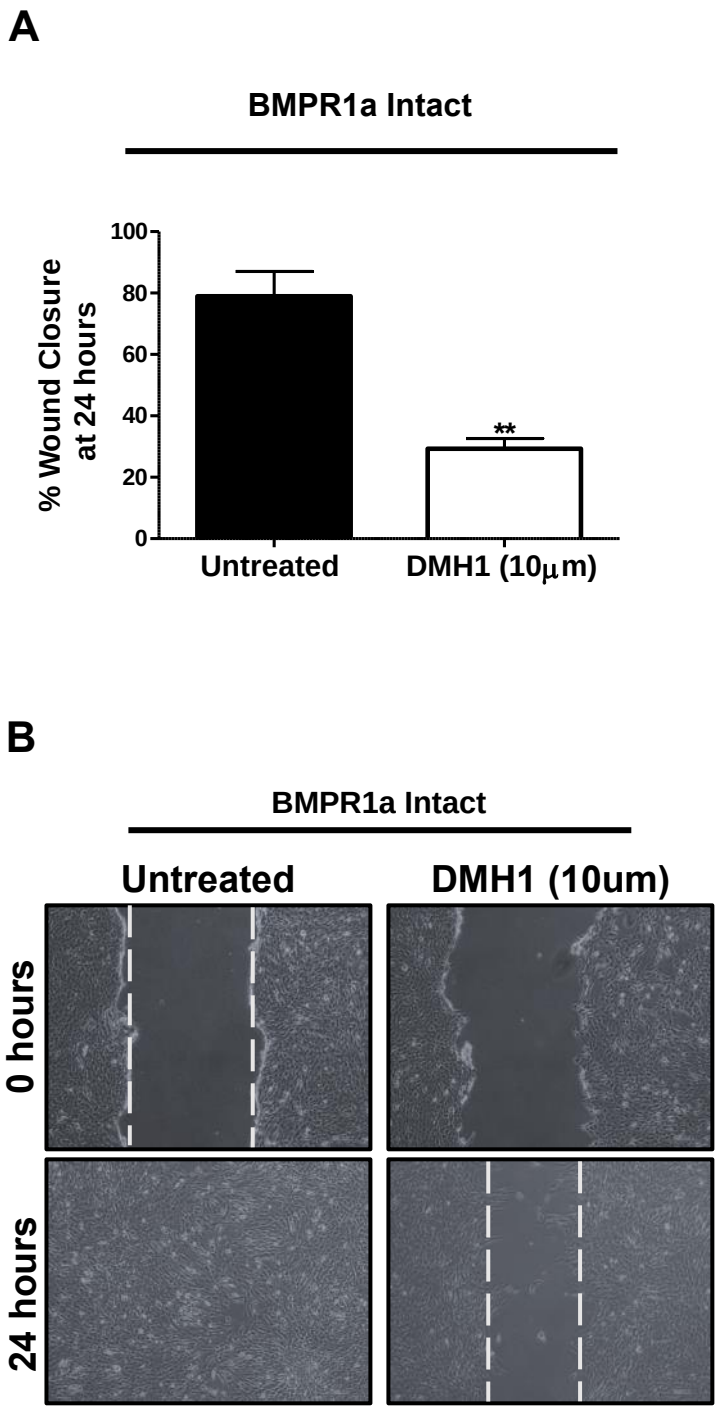


Figure 21. Pharmacological inhibition of BMP decreases proliferation and migration of oncogenic astrocytes

Figure 21. Pharmacological inhibition of BMP decreases proliferation and migration of oncogenic astrocytes

A,B) Treatment with DMH1 (10μM) significantly inhibited migration of transformed astrocytes as determined by a scratch assay. All cells were treated with Mitomycin-C (MMC) 2 hours prior to performing the scratch assay. Cells were treated with DMH1 at the time of the original scratch. B) Representative images of BMPR1a-intact astrocytes at time of the original scratch (0 hours) and at 24 hours (4x) with and without treatment of DMH1 (10μm). Significance was determined by a two-tailed Student's t-test. Bars indicate SEM. **p<0.01.

Malignant gliomas are highly invasive neoplasms, and infiltration of surrounding brain tissue contributes to tumor recurrence. Here we show that BMP signaling increases cell motility and the ability of astrocytoma cells to invade. These findings are consistent with studies of pancreatic and breast cancer cells[71,76]. In astrocytes, BMP signaling may be driving this effect through regulation of integrins as the reduction in cell mobility we observed in BMPR1a-KO cells was associated with a marked reduction in integrins beta 4 and 7 gene expression. Integrin beta 4 has been shown to be inversely correlated with survival in GBM[155].

BMP signaling is a well-known driver of astrocytic differentiation in normal neural stem/progenitor cells[57]. In the present study, abrogation of BMP signaling in transformed astrocytes was associated with increased gene expression of stem cell markers, including *Olig1*, *Olig2* and *Prom-1(CD133)*. In concordance with our findings, studies of neural progenitor cells have shown that BMP signaling suppresses the expression and activity of Olig1 and Olig2[31]. CD133, a cell surface antigen, has been touted as the primary marker of glioma stem cells, and BMP treatment has been shown to reduce the number of CD133-positive cells in glioma[78].

In addition, CD133-positive glioma cells may be resistant to radiotherapy, highlighting the importance of understanding the biology of CD133-expressing cells in glioma[34]. Our data suggest that the BMP pathway may be a mediator of CD133 expression in the more differentiated glioma compartment. The changes in stemness markers were not observed in the tumors, which may be a result of the type of cells that engrafted, effects of the tumor microenvironment, or differences in protein and gene expression.

Our *in vivo* model and *in vitro* studies suggest that pharmacological inhibition of BMP signaling could be a promising new therapeutic modality in HGG for the non-GSC component of the tumor, by reducing proliferation and invasion. Conceivably, the reduction in invasive properties of glioma cells would increase the effectiveness of standard therapies such as surgery and radiotherapy. Our studies also highlight the concept that the stem/progenitor-like

glioma compartment may respond differently than the bulk tumor to BMP-based and other therapies[78,79]. For example, when we treat human GBM cells grown as neurospheres with DMH1 we see no effect on proliferation supporting this hypothesis. This suggests that for effective treatment, multiple therapies may be required to target the differentiated and progenitor populations separately. Indeed, an understanding of the mechanisms that maintain glioma cells in more or less differentiated populations, while governing transitions from one compartment to the other, may lead to progress in glioma therapies.

CHAPTER IV: DISCUSSION

Since the implementation of Temozolomide in 2005, survival rates for GBMs have not significantly improved, with the median survival remaining at just over a year after diagnosis[156]. However, within the past fifteen years, significant gains have been made to our understanding of HGG including the discovery of glioma stem cells, defining the core genetic alterations and HGG molecular subtypes through the use of large-scale genomic and proteomic studies and the evolution of relevant and more informative animal models. We have taken advantage of these advances in the field to study BMP signaling in human HGG at the genetic and protein level, with the conclusion that BMP inhibition may be a novel therapeutic strategy in HGG.

BMPs comprise the largest subgroup within the TGFβ family[157]. It has been well established in the field of cancer research that TGFβs play dual tumor promoting and suppressing roles in cancer[65]. Recently it has become clear that BMPs have similar pro and anti-tumor effects in many cancers depending on the cellular and environmental context[66,67][**Figure 22**]. Our results indicate that this phenomenon is true within gliomas as well. To date, the most well characterized function of BMPs in gliomas is as a tumor suppressor on GSC. As first shown by Vescovi's group in 2006, BMP signaling promotes differentiation, suppresses proliferation and increases survival in murine models of HGG using GSC[78]. Since then BMPs have been shown to be one of the most effective inducers of differentiation in GSC. However, here we provide evidence that BMP signaling can be tumor promoting in gliomas depending on the cellular context.

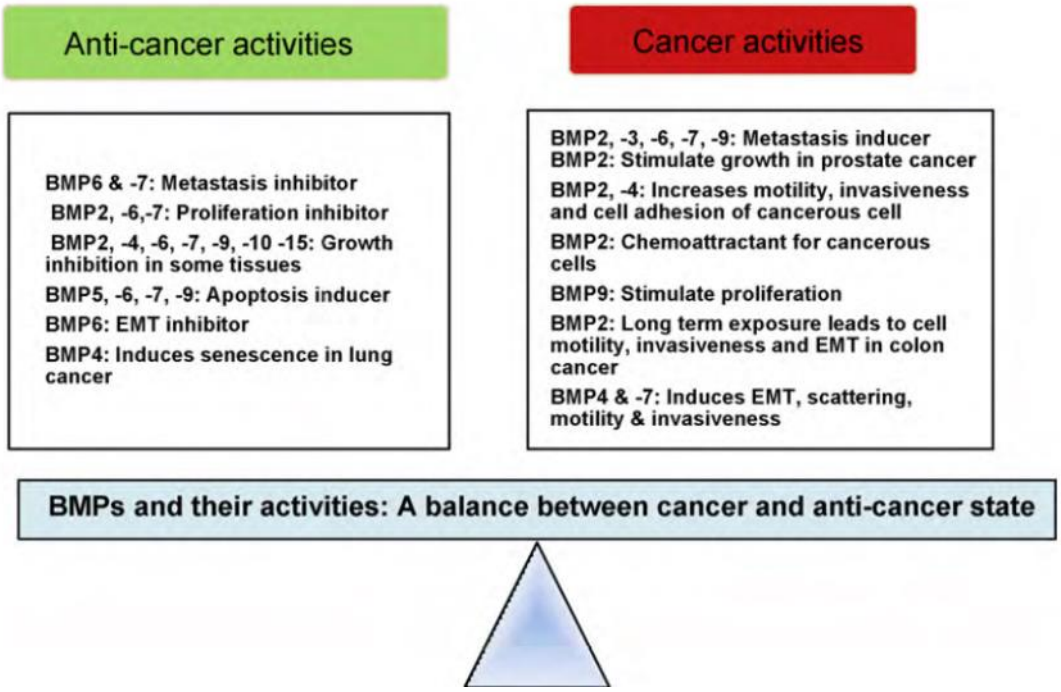


Figure 22. BMPs and their cancer and anti-cancer activities are described in a cancer cell

We hypothesize that there is a delicate balance between the cancer and anti-cancer environment of a cell. BMP molecules, depending on their environmental stimuli, can shift the delicate balance in either direction. Such a balance is necessary for the survival, normal growth, and development of a cell. Used with permission [67]

BMP SIGNALING IS ACTIVE AND RARELY ALTERED IN HUMAN HGG TUMOR CELLS

Several studies have examined the mRNA or protein expression of various individual BMP molecules in glioma datasets; however, we are the first to use genomic profiling to examine the BMP signaling family in a large dataset. In our analysis, we did not detect any recurrent mutations or deletions, suggesting that BMP signaling is largely intact and critical to the survival of tumorigenic cells. All mutations detected were found in only 1 or 2 patients and no alterations were found in more than 15% of patients. Additionally we found that certain BMP molecules are more highly expressed in PN tumors, similar to other published results [96,99]. As discussed earlier, MES tumors express neural stem cell markers such as CD133 and Nestin and PN tumors express markers of immature and developing neurons such as MAP2[16]. BMP signaling may be contributing to these signatures as a lack of BMP signaling in MES tumors may be driving a more stem-like phenotype and increased BMP signaling in PN tumors may result in an increase in differentiation. However, at this time, due to a lack of functional experiments, it remains unclear if BMP signaling is truly associated with a molecular subtype. The development of representative murine models for each HGG subtypes and additional collection of characterized human tumor cells will be critical to assess BMP signaling in each subtype. In addition, as more genomic data are collected and more tumors are characterized we will be able to make stronger conclusions regarding the expression of BMP molecules and if BMP signaling is associated with patient characteristics, treatments or HGG subtypes.

Parallel to our genomic analysis, we investigated BMP activity within HGG. Our analysis of p-Smad1/5/8 provides evidence that BMP signaling is active within the majority of HGG tumor cells. Our findings are in contrast to a previous publication which found the phosphorylation of Smads1/5/8 to be decreased in HGG compared to low grade gliomas and normal brain tissue [98]. Liu *et al* showed that decreased levels of p-Smad1/5/8 were correlated with decreased survival among all grades of gliomas and within GBMs, implying a tumor suppressive role for BMP signaling. However, there are several factors which should be considered when interpreting

these results. This study was done using 64 gliomas including 25 GBMs. Out of those 25 GBMs, only 5 patients showed high levels of p-Smad1/5/8. The expression of p-Smad1/5/8 was assessed using western blot quantification which limited their ability to assess the reduction in p-Smad1/5/8. Using western blot quantification it remains unknown if the reduction in expression is due to loss of signaling or reduced signaling. Additionally, tumor lysates generally include non-tumor tissue and infiltrates which may be contributing to the overall levels of p-Smad/1/5/8 observed. In our analysis, by using IHC, we were able to determine that BMP signaling in our tumor samples was active in approximately 70-90% of all tumor cells. In our study we did not observe a uniform decrease in p-Smad1/5/8 in HGG compared to non-tumor tissue. Furthermore, in 5 out of 30 GBM samples, we observed maximal staining intensity in 100% of tumor cells with patient survival ranging widely from 129 to 836 days. We were unable to make any significant associations between expression of p-Smad1/5/8 and survival due to the wide variation in intensity within all tumors analyzed. Our study contradicts that of Liu *et al* showing high intensity staining in HGG with no association with survival. Due to the conflicting results based on the limited number of patients analyzed in our study and by Liu *et al*, correlations of active BMP signaling in gliomas should be made using a larger number of patient samples of all tumor grades. Furthermore, additional downstream targets of BMP signaling, such as Id1, should be used to validate BMP activity.

BMP SIGNALING IN ONCOGENIC ASTROCYTES

In response to our results which showed high levels of BMP activity in HGG, we developed a novel murine model to investigate the role of BMP signaling on tumorigenic astrocytes. The results of our study described in chapter 3 illustrate that BMP signaling plays a tumor promoting role on tumorigenic astrocytes modulated through proliferation, migration and invasion. To our knowledge, our study is one of first few to examine BMP signaling in transformed astrocytes. Two other studies examining BMP signaling in oncogenic astrocytes

have been previously published, one which shows BMP signaling to be tumor suppressing and one showing BMP signaling to be tumor promoting[90,158]. In 2012 Liu *et al* showed that overexpression of BMPR1B in U87 and U251, human astrocytoma cell lines, decreased the growth of cells, increased apoptosis and increased the survival of mice in an intracranial injection model[158]. Upon overexpression of BMPR1B, the authors observed an increase in p21 and p27 and an increase in the number of cells found in the G0/G1 phase of the cell cycle, indicating that BMPR1B regulates cell cycle and arrests growth in human GBM astrocytes. Conversely, in 2014, Guo *et al* showed that micro-RNA suppression of BMPR1A led to decreased proliferation, invasion, migration and tumor growth in U87 cells, similar to our results [90]. In this paper, the authors found that treatment of U87 cells with BMP2 increased cell proliferation, migration and invasion and conversely, suppression of BMPR1A led to a decrease in the number of proliferating cells and an increase in the number of cells in the G1 phase of the cell cycle. When considered with our results, these studies suggest the downstream effects of BMP signaling may differ based on the type I receptor through which the signal is propagated.

This hypothesis is supported by previous studies which show that BMPR1A and BMPR1B have different and non-overlapping effects during development and astrogliosis [159,160]. BMPR1A homozygous deletion is embryonic lethal in mice showing no formation of the mesoderm, whereas homozygous deletion of BMPR1B in mice results in no obvious neural defects[64,101]. Conditional knockout models have shown that loss of BMPR1A results in abnormal early forebrain development, loss of the choroid plexus and abnormal oligodendrocyte formation in the brain[153]. As discussed in the introduction, BMPR1A and BMPR1B act sequentially during development and each play specific roles in the regulation of neural progenitor cells. After development, the type I receptors continue to have specific, different roles within the brain. Sahni *et al* showed that during astrogliosis, following a spinal cord injury, BMPR1A and BMPR1B had directly opposing effects. Using a conditional knockout mouse model, loss of BMPR1A resulted in defective gliosis and loss of BMPR1B led to increased

gliosis[160]. In the context of gliomas, BMPR1A may play a dominant tumor promoting role when present and BMPR1B may play a tumor suppressing role when expressed more highly than other type I receptors, such as in the absence of BMPR1A or overexpression of BMPR1B.

These varying effects may in part be mediated by ligand binding to the receptors. Studies have shown that BMPs 2 and 4 preferentially bind to BMPR1A[39] and BMPs 5,6,7 and 8 preferentially bind to BMPR1B[161]. In brain tumors and other types of cancer, BMP2 has been shown to have a tumor promoting effect[39,90]. Additionally, Wu's group showed that increased BMP2 expression was associated with shorter survival of glioma patients and was a significant predictor of survival in GBMs[93]. Therefore, it is possible that within gliomas, BMP2 and 4 primarily signal through BMPR1A inducing tumor promoting effects, whereas ligands signaling through BMPR1B propagate tumor suppressing effects. One interesting study that could be done to address this question would be to generate a knockout model of the BMPR1B receptor in the context of p53 loss and Kras activation. Using an identical genetic background, we would be able to directly compare the effects of BMPR1A or BMPR1B loss on tumor formation. Additionally*, in vitro* experiments should be conducted to compare the effects of varying BMP ligands, such as BMP2 and 7, on proliferation, migration and invasion in GBM astrocyte cells.

Further characterization of BMPR1A in HGG is also needed to completely understand its tumor promoting role on tumor formation and growth. In our study, we were unable to determine if the decreased tumor growth we observed was due to lack of engraftment, increased apoptosis or decreased cell proliferation. In our experimental model we were unable to detect tumor growth until the tumor had progressed to an aggressive, high grade tumor, often necessitating euthanasia of the mouse. Other methods that could be used in future studies include the use of bioluminescence imaging or magnetic resonance imaging (MRI) to observe cell survival upon engraftment and the growth rate of the tumors over time. By histological examination we saw no difference in proliferation or apoptosis in the tumors; however, we only looked at the end time point when the tumors were aggressive enough to necessitate

euthanasia of the mice. Future studies should include additional time points to observe both the tumor growth and histological characteristics of the tumors over time. Additionally, further studies are needed to determine the downstream mechanism by which BMP signaling regulates cell proliferation and migration. Upon knockout of BMPR1A we investigated the mRNA expression of several genes through which TGFβ or BMPs are known to regulate cell proliferation in gliomas or other systems[162–164] and saw no significant change in p21, p27 or p57 expression. Further analysis will need to be done to determine the mechanism by which loss of BMP signaling decreases proliferation. Similarly, we investigated a panel of genes associated with migration and invasion and found that our BMPR1a KO astrocytes showed a near or complete loss of integrin beta 4 and 7. Rescue studies should be executed to determine if loss of these integrins is responsible for the decrease in migration and invasion we observed.

Finally, one of the most interesting outcomes of our study is the finding that BMP signaling regulates neural progenitor cells and differentiated astrocytes fundamentally differently. As discussed previously, many publications have shown BMP activity to be tumor suppressing on the population of GSC, primarily through increased differentiation and decreased proliferation. Here we show evidence that BMP signaling has opposite effects on the astrocytic population. Our preliminary data show that BMP inhibition has different effects on murine progenitors and astrocytes with identical genetic backgrounds. Similarly, BMP inhibition has different effects on human GSC and astrocyte populations. Our study highlights the importance of understanding the complexity of the BMP pathway in gliomas. While BMP treatment has been suggested as a source of therapy, our results show that this may actually act as a tumor promoter on the majority of the cells within the tumor. Several publications have investigated forms of BMP therapy including microspheres designed to release BMP ligands and an oncolytic vaccinia virus overexpressing BMP4, which all showed a survival benefit in murine models[78,87,88]. While these treatments were effective in murine models created from GSC, our results suggest that in the clinic, treatment of the whole tumor with BMP ligands would

be detrimental to patient survival. In contrast to previous studies, we suggest the use of BMP inhibition as a novel source of therapy.

THERAPEUTIC INHIBITION OF BMP SIGNALING

Inhibition of BMP signaling has been shown to be beneficial in models of breast, ovarian and lung cancer[74,76,154,165]. Strategies used to inhibit BMP signaling have included soluble antagonists or neutralizing antibodies[166]. While these inhibitors showed beneficial effects, they were limited by short half-lives and gain of function mutations downstream in the BMP pathway. In order to more specifically target BMP signaling and successfully inhibit BMP signaling *in vivo*, small molecule inhibitors have recently been developed to specifically target BMP type I receptors[149,167,168]. The first small molecule inhibitor discovered for BMP signaling was Dorsomorphin, identified in 2008 as a compound capable of inhibiting BMPR1A, BMPR1B and ACVR1[168]. However, Dorsomorphin was found to inhibit AMPK and VEGF signaling as well. Over the past five years several more inhibitors of BMP type I receptors have been developed to increase specificity and reduce off target effects[149]. One specific inhibitor, dorsomorphin homologue 1, (DMH1), has been shown to specifically target BMPR1A, BMPR1B and ACVR1 without detectable inhibition of TGFβ, AMPK or VEGF signaling [149]. DMH1 inhibits ACVR1 and BMPR1A most strongly with inhibition of BMP signaling in mouse mesenchymal cells through ACVR1 and BMPR1A at an IC50 of less than 50uM. In our studies we found that in murine and human transformed astrocytes, DMH1 significantly inhibited BMP signaling at concentrations as low as 10μM with no toxicity at concentrations up to and including 100μm. Following these discoveries, we and others have shown that BMP inhibitors, specifically DMH1, can act as a tumor suppressing agent.

We showed that in a Polyoma T mouse model of breast cancer, DMH1 was shown to inhibit tumor growth and metastasis. Mice receiving treatments of DMH1 through an osmotic pump showed a significant decrease in the number of primary tumors that formed and a

significant decrease in pulmonary metastases. In addition, the treated tumor cells showed evidence of increased apoptosis and decreased proliferation [76]. DMH1 has also been shown to have similar tumor suppressing effects on non-small cell lung cancer cells (NSCLC). The BMP pathway is an attractive target for treating NSCLC as overexpression of BMP2 is associated with NSCLC with little to no BMP activity in normal lung tissue. In addition, a previous study showed that treatment of NSCLC with Noggin suppressed tumor growth. In a similar manner, NSCLC treated with DMH1 had decreased proliferation, migration and invasion and decreased tumor growth in a xenograft model[154].

In a series of *in vitro* assays we investigated the effects of DMH1 on ovarian cancer cell lines. Ovarian cancer cells have high endogenous levels of active BMP signaling and BMP signaling has been shown to play tumor promoting roles [69,169]. We discovered that inhibition of BMP signaling by DMH1 led to decrease in tumor sphere growth and enhanced the sensitivity to Cisplatin treatment[74]. Similarly, a different study showed that treatment of ovarian cancer cells with Dorsomorphin or LDN193189, a Dorsomorphin derivative, blocked cell cycle progression, reduced cell migration, increased survival in a xenograft model and resensitized cells to carboplatin[169].

Similar to the studies discussed above, here we present BMP inhibition as a treatment in HGG. As part of a multi-modal treatment regimen, BMP inhibition may slow the growth and invasion of oncogenic astrocytes making tumors more susceptible to the current therapies such as surgery and radiation [**Figure 23**].

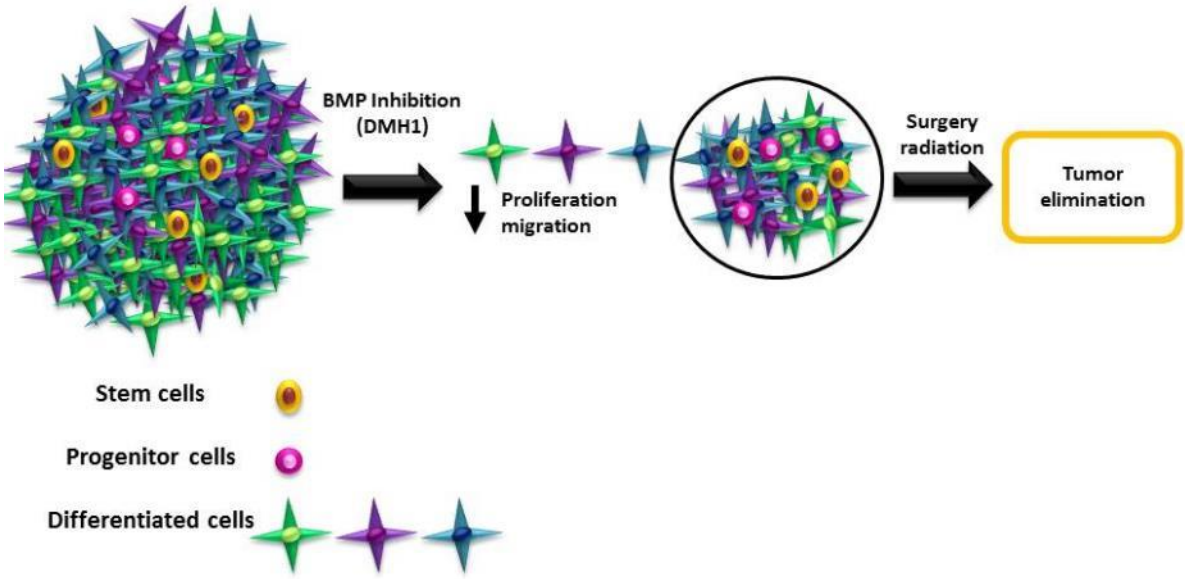


Figure 23. BMP inhibition in HGG

A HGG is a heterogeneous tumor comprised of many differentiated tumor cells and a small population of cells with progenitor and stem-like qualities. Inhibition of the BMP pathway leads to decreased proliferation and migration of the bulk of the tumor enhancing the ability of the current therapeutics such as surgery and radiation to eliminate the tumor.

In addition to adult HGG, BMP inhibition is a very exciting target for a rare, but deadly pediatric glioma. In 2014 it was discovered that activating mutations in activin A receptor type I (ACVR1) are present in 20-30% of patients with diffuse intrinsic pontine glioma (DIPG). DIPGs represent approximately 10% of all pediatric CNS tumors, most commonly diagnosed between ages of 5-9. This aggressive tumor is uniformly fatal with a median survival of less than one year from diagnosis[170]. Four groups identified 6 recurrent mutations in ACVR1, resulting from amino acid substitutions within the glycine-serine or kinase subdomain of ACVR1 shifting the kinase into the active conformation [171–174]. A gain of function has been demonstrated by increased phosphorylation of Smads1/5/8 and increased expression of Id1[170]. Additionally, transfection of brainstem progenitor cells with mutant ACVR1 led to an increase in cell proliferation[175]. Within pediatric gliomas, ACVR1 mutations are exclusively found in DIPG[176]. These mutations have been observed in biopsies collected at the time of diagnosis, suggesting ACVR1 mutations are present during the initial phases of tumor growth[175]. Interestingly four of the same activating mutations in ACVR1 were first identified in Fibrodysplasia ossificans progressiva (FOP), a rare genetic disorder in which muscle is converted into bone leading to asphyxiation and death [171,177,178]. It has been understood for about a decade that FOP results from activating mutations within the intracellular domain of ACVR1[177]. As a result of this recent discovery, targeting BMP type I receptors, specifically ACVR1, presents a novel potential therapeutic target for both DIPG and FOP patients [**Figure 24**].

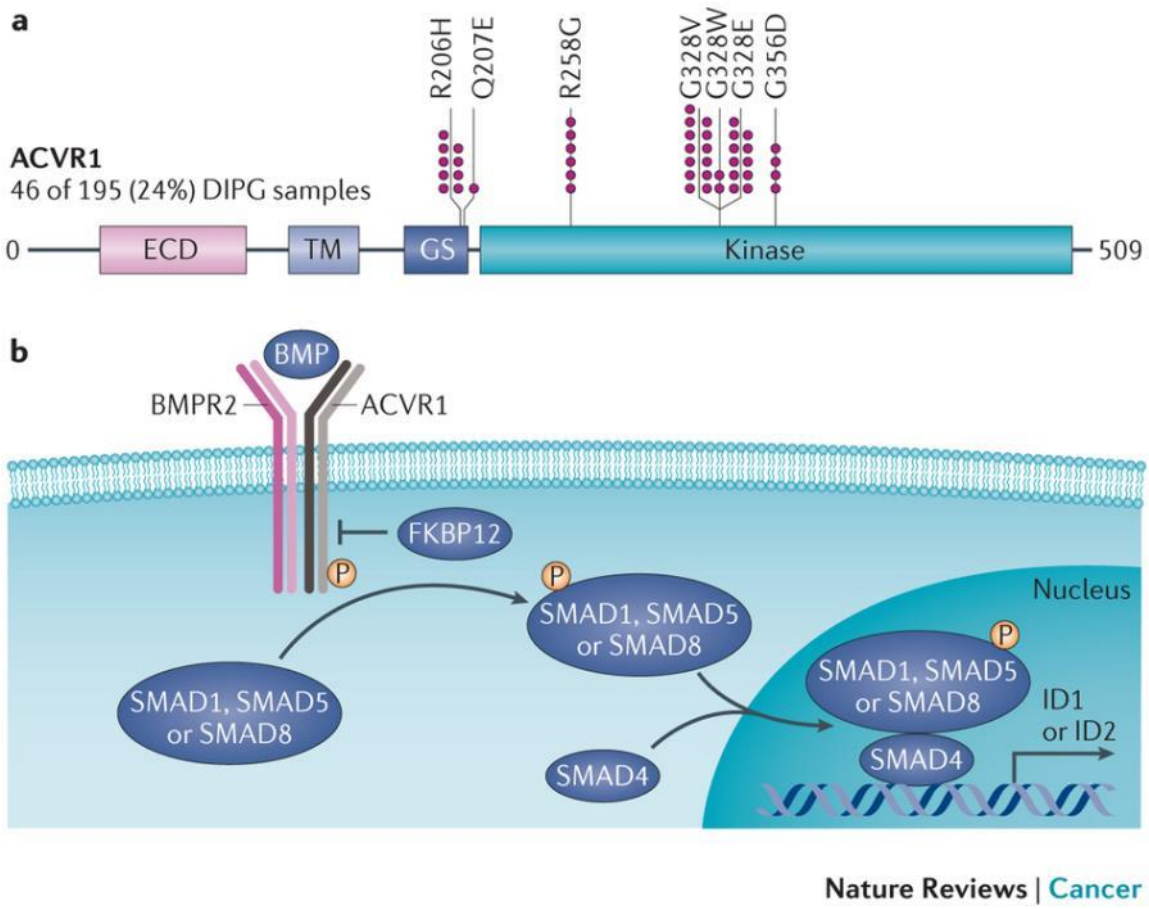


Figure 24. Activin receptor type 1 (ACVR1) mutations in diffuse intrinsic pontine glioma (DIPG)

a) Cartoon showing somatic mutations in the glycine/serine-rich inhibitory domain (GS) and kinase domains of ACVR1 in 46 of 195 (24%) DIPG samples. No mutations were identified in the extracellular (ECD) or transmembrane (TM) domains. All residues targeted are common to those observed in the germlines of patients with fibrodysplasia ossificans progressiva (FOP), with the same amino acid substitutions, except for R258G and G328V, which are, to date, unique to DIPG. b) Bone morphogenetic protein (BMP) signaling pathway. Upon ligand binding, a type 2 receptor, such as BMPR2, heterodimerizes with and phosphorylates (P) ACVR1, a serine/threonine kinase, which in turn phosphorylates the transcription factors SMAD1, SMAD5 or SMAD8, causing binding to SMAD4, translocation to the nucleus, and transcription of target genes such as inhibitor of DNA binding 1 (ID1) and ID2. The ACVR1 mutations in DIPG and FOP enhance the kinase function and/or disrupt the binding of the negative regulator the 12kD FK506 binding protein (FKBP12) to ACVR1, conferring activation of this pathway. Used with permission[170]

CONCLUDING REMARKS

The role of BMP signaling in gliomas is a topic which has only recently been examined in depth and remains incompletely understood. BMPs have generally been thought of as tumor suppressors; however, recently we and others have begun to show that BMP signaling can also be tumor promoting. As members of the TGFβ family, it is unsurprising that BMPs play a variety of roles within a complex and heterogeneous tumor. While it is still unclear all the various ways BMP signaling affects tumorigenesis, our research enhances the field of glioma research and emphasizes the importance of understanding the various roles of this morphogen family. My ultimate hope is that our discoveries will contribute to future research to find ways to manipulate BMP signaling to benefit glioma patients.

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