Growth-Inhibitory Activity of Bone Morphogenetic Protein 4 in Human Glioblastoma Cell Lines Is Heterogeneous and Dependent on Reduced SOX2 Expression

Erika Dalmo, Patrik Johansson, Mia Niklasson, Ida Gustavsson, Sven Nelander, and Bengt Westermark

ABSTRACT

Glioblastoma multiforme continues to have a dismal prognosis. Even though detailed information on the genetic aberrations in cell signaling and cell-cycle checkpoint control is available, no effective targeted treatment has been developed. Despite the advanced molecular defects, glioblastoma cells may have remnants of normal growth-inhibitory pathways, such as the bone morphogenetic protein (BMP) signaling pathway. We have evaluated the growth-inhibitory effect of BMP4 across a broad spectrum of patient samples, using a panel of 40 human glioblastoma initiating cell (GIC) cultures. A wide range of responsiveness was observed. BMP4 sensitivity was positively correlated with a proneural mRNA expression profile, high SOX2 activity, and BMP4-dependent upregulation of genes associated with inhibition of the MAPK pathway, as demonstrated by gene set enrichment analysis. BMP4 response in sensitive cells was mediated by the canonical BMP receptor pathway involving SMAD1/5/9 phosphorylation and SMAD4 expression. SOX2 was consistently downregulated in BMP4-treated cells. Forced expression of SOX2 attenuated the BMP4 sensitivity including a reduced upregulation of MAPK-inhibitory genes, implying a functional relationship between SOX2 downregulation and sensitivity. The results show an extensive heterogeneity in BMP4 responsiveness among GICs and identify a BMP4-sensitive subgroup, in which SOX2 is a mediator of the response.

Implications: Development of agonists targeting the BMP signaling pathway in glioblastoma is an attractive avenue toward a better treatment. Our study may help find biomarkers that predict the outcome of such treatment and enable stratification of patients.

Introduction

Research on the biology of glioblastoma multiforme (GBM) has progressed considerably during the last decade. Aberrations in four main signaling pathways (Ras-Erk, pRB, p53, and PI3K-AKT) are common themes (1, 2). Unfortunately, basic information on the molecular defects in GBM has not been translated into therapy. GBM is still treated by relatively unsophisticated methods: surgery, radiotherapy, and chemotherapy. There are two main reasons why this rather harsh treatment fails in virtually all cases. First, tumor growth is fueled by glioma-initiating cells (GIC), which have stem cell characteristics (3, 4) and as such resist therapy (4, 5). Second, GBM cells are extremely invasive and, at the time of diagnosis, tumor cells have, in principle, invaded the whole brain. Needless to say, there is an urgent need for better therapy.

An alternative to targeting oncogenic drivers in GBM, such as receptor kinases, would be to harness remnants of normal growth regulatory pathways. In this context, bone morphogenetic proteins (BMP) are of interest. BMPs belong to the TGF beta superfamily and have pivotal roles in mammalian development, including the central nervous system (6). Piccirillo and colleagues (7) showed that even a short preexposure of BMP4 to human GICs was sufficient to inhibit tumor formation in mice. Although a study by Carén and colleagues (8) failed to reproduce these results, a partial to complete reprogramming of GICs to an astrocyte-like phenotype was observed upon prolonged BMP4 exposure. Another striking observation was that the response to BMP4-mediated growth inhibition varied among the small number of cell lines that were included in the study.

Our laboratories have launched the Human Glioma Cell Culture (HGCC) resource as a publically available repository of a large number of annotated, short tandem repeat (STR)-profiled and phenotypically characterized GBM cell lines (hgcc.se; ref. 9). In this communication, we have assessed the potential of BMP4-mediated growth inhibition in a panel of 40 human GBM cell lines, to scrutinize the possibilities and limitations of BMP4-based treatment across a large spectrum of patients. The objectives of our investigation were to study the effects of BMP4 across a large number of GBM cell lines, and to find molecular markers distinguishing high-sensitive from low-sensitive lines to better understand mechanisms mediating BMP4-induced growth regulation of glioblastoma.

Our results demonstrate an extensive diversity in BMP4 response between GBM cells. Downregulation of SOX2 was shown to have a causative role in BMP4-mediated growth inhibition. Furthermore, BMP4 sensitivity was associated with an upregulation of genes involved in negative regulation of the MAPK signaling pathway.

Materials and Methods

Cell culture

Human GBM cell lines were obtained from the HGCC resource (hgcc.se; ref. 9). The authenticity of the cell lines (passage 4 and 50) was...
established by STR genotyping performed using the AmpF/STR Identifier PCR Amplification Kit (Applied Biosystems). All cell lines were tested for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Cells were cultured adherently on Primaria dishes (BD Biosciences) coated with mouse laminin (Sigma-Aldrich) in serum-free Neurobasal and DMEM/F12 media (1:1) supplemented with N2 and B27 (Thermo Fisher Scientific) and human recombinant FGF2 and EGF, 10 ng/mL (PeproTech). For cell detachment, StemPro Accutase or TrypLE Select (Thermo Fisher Scientific) was used.

**Proliferation experiments**

For BMP4 resistance experiments, cells were seeded in 24-well format with 3,000 cells/well at day 0, and treatment started after 24 hours. Medium was replaced every second to third day with new medium containing 10 ng/mL BMP4 (Thermo Fisher Scientific). Relative number of cells was determined at day 1, day 7, and day 15 using AlamarBlue (Thermo Fisher Scientific) according to the manufacturer’s protocol. Biological quadruplicates were used for each cell line and time point.

EdU incorporation assays were performed using the Click-IT EdU Alexa Fluor 488/555 Imaging Kit (Thermo Fisher Scientific). EdU-incorporating cells were detected using Click-IT EdU detection buffer and imaged with a fluorescence microscope. Quantification was performed using ImageJ software (10). All proliferation experiments were performed with cells between passage 4 and 39.

**SOX2 overexpression**

The lentiviral vectors EX-T2547-Lv202 and EX-NEG-Lv202 (Gen-eCopoeia Inc) were used for Sox2 overexpression and as control, respectively. Each vector was cotransfected with plasmids pLP1, pLP1, eCopoeia Inc) were used for Sox2 overexpression and as control, respectively. Each vector was cotransfected with plasmids pLP1, pLP1, and VSVg (5 μg/mL of each; Thermo Fisher Scientific) into HEK293T packaging cells for virus production. The virus supernatant was collected at 48 and 72 hours after transduction and virus was purified by ultracentrifugation at 20,000 rpm for 2 hours and stored in aliquots at −80°C until use. Virus titer was determined using the qPCR Lentivirus Titration (Titer) Kit (Applied Biological Materials) according to the manufacturer’s instructions.

For stable overexpression, 300,000 cells were infected with lentivirus at a multiplicity of infection (MOI) of approximately 6 in suspension for 4 hours. The cells were seeded onto laminin-coated dishes and virus washed off after 24 hours. 72 hours after transduction, puromycin (0.5 μg/mL, Sigma-Aldrich) was added to the medium and cells were kept under selection throughout experiments.

**Western blot analysis**

Cells were lysed (1% SDS and 10 mmol/L Tris-HClpH 7.5; or RIPA lysis buffer containing cOmplete protease inhibitor and PhosStop phosphatase inhibitor (Roche)) and protein concentration was determined (Pierce BCA Protein Assay Kit, Pierce). Proteins were separated on a 4%–12% Bis-Tris polyacrylamide gradient gel (NuPAGE, Thermo Fisher Scientific) under reducing conditions and transferred onto a nitrocellulose membrane (Novex, Thermo Fisher Scientific), followed by blocking (1%–5% BSA or milk) and primary antibody incubation at 4°C overnight. After horseradish peroxidase–secondary antibody (GE Healthcare) incubation, membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) or ECL Select WB Detection Reagent (GE Healthcare) and visualized using an ImageQuant Las 4000 (GE Healthcare). 0.4 M NaOH was used to strip the filters before reblooming. Primary antibodies: rabbit anti-Sox2 (AB5603, Millipore), rabbit anti-phospo-SMAD1/5/9 (catalog no. 13820, Cell Signaling Technology), goat anti-SMAD1 (AF2039, R&D Systems), rabbit anti-phospo-SMAD5 (catalog no. 9517, Cell Signaling Technology), rabbit anti-GFP (ab290, Abcam), mouse anti-β-actin (A5441, Sigma-Aldrich), rabbit anti-cyclophilin B (catalog no. 43603, Cell Signaling Technology). Quantification was performed using Image Studio Lite version 5.2 (LI-COR Biosciences).

**Quantitative RT-PCR**

RNA was extracted with the RNeasy Mini Kit (Qiagen), followed by removal of genomic DNA with DNase I, RNase-free (Thermo Fisher Scientific), and reverse transcription with the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Quantitative PCR was performed using CybrGreen (Thermo Fisher Scientific) with a StepOnePlus Real Time PCR system (Applied Biosystems) and expression changes calculated with the ΔΔCt method using GAPDH as endogenous control. Primer sequences: ID1 Forward: TTGCCCCATTCGTGTTCAGGC, ID1 Reverse: CG-TAGACGACAGCGTGTACC, ID3 Forward: GTTGCCCTGACAC-CTCCGAAAC, ID3 Reverse: CTCCTCAGGCCACAGTTCCAC, SMAD4 Forward: AAAAGCGCCATCTTCCAGCAC, SMAD4 Reverse: AGGCCAGTAAAGTTGCGGGA.

**siRNA experiments**

Cells were seeded one day prior to siRNA transfection and the cells were incubated with the siRNA and Lipofectamine RNAiMAX (Thermo Fisher Scientific) overnight. Silencer select siRNA’s (Thermo Fisher Scientific): Negative control catalog no. 4390843, siSMAD4: catalog no. 4390824, ID 8403.

**RNA sequencing**

RNA was extracted (RNeasy Mini Kit, Qiagen) and the sequencing library prepared using TruSeq stranded total RNA Library Preparation Kit (Illumina) with ribosomal depletion using RiboZero Gold (Epicentre). Libraries were paired-end sequenced for 50 cycles in one NovaSeq SP lane on a NovaSeq 6000 sequencing system. Raw read pairs were aligned to the human genome (hg19 build) using HISAT2 (11; Galaxy Version 2.1.0), and read counts for each gene were determined with featureCounts (12; Galaxy Version 1.6.3). For multifactor differential expression analysis, the edgeR package was used (13; version 3.22.5) within the R software version 3.5.0 (http://www.r-project.org). Data are available via NCBI Gene Expression Omnibus (GSE143934).

**Statistical analysis and visualization**

Pearson correlations, Spearman correlations, and χ2 tests were calculated using R software. Other statistical tests were performed with GraphPad Prism software (GraphPad Software, Inc.). Proliferation curves, scatterplots, Venn diagrams, and heatmaps were generated in R. Box plots were visualized in GraphPad Prism.

**Gene set enrichment analysis**

Pearson correlations between BMP4 resistance score and basal gene expression for all 40 HGCC lines were used for ranking genes with the GSEA software (14). One thousand permutations of the phenotype value were performed and the weighted enrichment statistic was used. Single sample gene set enrichment analysis (ssGSEA) was performed as described previously (15).
For RNA sequencing data, a preranked gene list was used with 
\(-\log_{10}(P)/\log_{10}(fold\ change)\) as ranking in the GSEA software. Analysis of gene ontology (GO) terms for overlapping genes was performed using the Database for Annotation, Visualization, and Integrated Discovery (16, 17).

Results

Human GBM cell lines display a wide range of response to BMP4 exposure

Forty cell lines from the HGCC repository representing all GBM molecular subtypes (9) were analyzed with regard to BMP4-induced growth inhibition. Cell density was determined after 7 and 15 days of culture in the presence or absence of BMP4. A wide range of responses to BMP4 treatment was observed, from virtually no effect on cell proliferation to an almost complete inhibition (Fig. 1A). The difference in increase in cell number from day 7 to 15 between BMP4-treated and control cells was used to construct a BMP4 resistance score. A connection between high activation of a mesenchymal signature and BMP4 resistance could be seen (Fig. 1B).

To get a global view of how long-term BMP4 exposure affects sensitive and resistant cells, RNA sequencing was performed on two sensitive (U3017 and U3065) and two resistant (U3028 and U3019) cell lines cultured in the absence or presence of BMP4 for 14 days (Fig. 1C). GSEA was used to identify gene sets that were differentially regulated depending on the BMP4 resistance status. Notably, MAPK-inhibitory genes were more upregulated in sensitive cells than in resistant cells upon BMP4 exposure (Fig. 1D).

BMP4 induces canonical signaling both in sensitive and resistant GBM cell lines

A potential mechanism by which GBM cells may escape the growth-inhibitory signal of BMP4 is to silence components of the canonical signaling pathway, that is, canonical receptors, R-SMADs, or the co-SMAD SMAD4 (Fig. 2A). To assess whether this is a major resistance mechanism, we analyzed transcriptome data available from the GBM cell lines (9) and plotted mRNA expression levels of the signaling components against the BMP4 resistance score. The expression of the type II receptor ACVR2B mRNA displayed a negative correlation with BMP4 resistance (Pearson \(r = -0.56\)) but no other general pattern could be observed (Fig. 2B). However, the two highly resistant lines U3054MG and U3019MG had among the lowest expression of several signaling components: BMPR1A, ACVR2B, SMAD4, and SMAD1 for U3054MG and BMPR1A, BMPR2, ACVR2A, ACVR2B, SMAD1, and SMAD5 for U3019MG (Fig. 2B).

To evaluate whether the relatively low mRNA expression levels of BMP4 signaling components would lead to failure to activate canonical BMP4 signaling, we exposed U3054MG and U3019MG to BMP4 and analyzed SMAD1/5/9 phosphorylation. In addition, the resistant cell line U3028MG and two sensitive lines, U3017MG and U3056MG, were evaluated for comparison. In all lines analyzed, BMP4 induced phosphorylation of SMAD1/5/9 (Fig. 2C). Thus, despite the relatively low mRNA levels, protein levels of the signaling components are evidently high enough also in the resistant lines to provide a functional signaling cascade.

Known signaling events downstream of BMP4 include upregulation of the inhibitor of DNA-binding (ID) genes ID1 and 3 (18). We proceeded to stimulate five sensitive and five resistant GBM cell lines with BMP4. Upregulation of ID genes was found in all cell lines and the degree of upregulation did not correlate with resistance (Fig. 2D). This finding strengthens the notion that BMP4 resistance cannot be explained by nonfunctional BMP signaling.

BMP4 has been shown to signal through noncanonical signaling via MAPK (19). To investigate whether noncanonical signaling mediates the BMP4 response in GBM cells, we used siRNA against SMAD4 to disrupt canonical signaling in the most sensitive line, U3017MG. During canonical signaling, the co-SMAD SMAD4 is needed for the formation of the SMAD-complex and the successive translocation into the nucleus for transcriptional regulation of BMP target genes (20). SMAD4 mRNA could successfully be reduced down to 25%, resulting in a reduced ability to upregulate ID1 and ID3 upon BMP4 exposure (Fig. 2E). In addition, the effect on BMP4-induced growth inhibition was lost (Fig. 2F). Thus, disruption of the canonical signaling does not lead to a compensatory activation of noncanonical signaling.

BMP4-sensitive GBM cell lines display a stem cell-like signature and high SOX2 activity

To identify potential general cellular processes and transcriptional programs related to BMP4 responsiveness, we examined global mRNA expression data in relation to the BMP4 resistance scores. A heatmap of the expression profile of top 20 genes associated with BMP4 response (both sensitivity- and resistance-related) across 40 GBM cell lines is shown in Fig. 3A. No BMP-related genes were found. One of the genes associated with sensitivity was ASCL1, which has been implicated in maintaining GBM stem cell properties in a Wnt-dependent manner by upregulating DKK1 (21). Intriguingly, DKK1 together with FZD2, another negative regulator of Wnt signaling, were among the 20 genes correlating with resistance. In addition, GSEA showed that genes coding for components along the Wnt canonical pathway correlate with BMP4 sensitivity (PID_WNT_CANONICAL_PATHWAY, NES = \(-1.60, P = 0.02\)).

In line with the results from above (Fig. 1B), GSEA revealed a link between BMP4 sensitivity and expression of genes typical of the proneural GBM molecular subtype, whereas BMP4 resistance was associated with the expression of mesenchymal GBM molecular subtype genes (Fig. 3B). Patel and colleagues (22) showed that proneural GBM cells display a more stem-like signature than mesenchymal cells, suggesting that the proneural/mesenchymal axis reflects a stemness/differentiation scale. We applied the stemness signature of Patel and colleagues to interrogate the transcriptomes of our panel of GBM cell lines in relation to the BMP4-resistant scores, and found that it was strongly associated with BMP4 sensitivity (\(P = 0.002\), Fig. 3C).

To validate this finding, we used the gene expression signatures from Ben-Porath and colleagues (23). Here, poorly differentiated tumor types were associated with embryonic stem (ES) cell signatures along with high expression of transcription factors SOX2, MYC, NANOG, and OCT4 targets and low expression of polycomb targets. Interestingly, all ES cell and transcription factor gene sets were enriched in BMP4-sensitive GBM cell lines, while polycomb target genes displayed the opposite pattern, that is, were enriched in the resistant GBM cell lines (Fig. 3D). Furthermore, analysis of gene sets from Singh and colleagues (24) showed that transcripts negatively regulated by SOX2 have a tendency to be enriched in resistant lines while positively regulated transcripts are significantly connected to BMP4 sensitivity (Fig. 3E). Altogether, our results strongly suggest that sensitive cell lines display a stem-like signature and have a high SOX2 activity compared with resistant ones. We therefore focused our study on the role of SOX2 in the regulation of BMP4 responsiveness.
High SOX2 expression is connected to sensitivity and BMP4 downregulates SOX2 in sensitive lines

Previous studies have implicated SOX2 in maintaining stemness properties of GBM cells (25, 26). As shown in Fig. 4A, higher expression levels of SOX2 mRNA were correlated with a stronger BMP4 response. Previous studies of BMP4-treated GBM cells at the single-cell level have revealed a robust downregulation of SOX2 (27). As expected, SOX2 protein levels were markedly reduced in BMP4-sensitive cells (U3008MG, U3017MG, and U3065MG) after 7 days of exposure (Fig. 4B). Out of the three resistant cell lines tested, only one showed a robust SOX2 downregulation (U3028MG) while the others either displayed only a modest downregulation (U3019MG) or had nearly undetectable SOX2 protein (U3054MG).

Forced expression of SOX2 attenuates BMP4-induced growth retardation

We asked the question whether SOX2 downregulation has a causative role in the BMP4-mediated growth inhibition. We stably transduced the six cell lines with lentiviral Myc-tagged SOX2 to provide cells with a SOX2 source that could not be transcriptionally downregulated upon BMP4 stimulation (Fig. 5A). With a MOI of 6, a vast majority (>99%) of cells were expected to receive between 1 and 12 lentiviral particles.

Figure 1.
Human glioblastoma initiating cells have a variable response to BMP4-mediated growth inhibition. A, Proliferation curves for 40 GBM cell lines cultured at 10 ng/mL BMP4 for 15 days. The number below each curve denotes the BMP4 resistance score, which is based on the cell number increase from day 7 and day 15. Error bars, SD, n = 4. B, Box plot with BMP4 resistance scores (left) and corresponding –log10(P) values of raw ssGSEA enrichment scores for each GBM molecular subtype, shown as a heatmap (right) for each cell line. PN, proneural; CL, classical; and MS, mesenchymal. C, Schematic overview of cells selected for RNA sequencing. Cells were cultured with BMP4 for 14 days. D, Top six enriched GSEA GO signatures differing between sensitive (U3017MG, U3065MG) and resistant (U3019MG, U3028MG) cell lines after 14 days of BMP4 treatment.
Canonical BMP4 signaling is functional in both resistant and sensitive cell lines, and is needed in a sensitive line to mediate BMP4 response. Figure 2. Overview of the BMP4 signaling pathway, highlighting the genes encoding the canonical signaling components. B, Scatter plots of baseline expression levels of BMP4 signaling components plotted against BMP4 resistance score. The resistant cell lines U3054MG (gray) and U3079MG (red), and the sensitive cell lines U3017MG and U3056MG (blue) are circled. C, Western blot analysis of phospho-SMAD1/5/9 activation in two sensitive and three resistant lines at 1 and 6 hours ± BMP4 (10 ng/mL). Total SMAD 1 and SMAD 5 levels are shown. Relative phospho-SMAD1/5/9 levels are depicted below the cyclophilin B loading control. The same blot was probed repeatedly with the antibodies used. Sensitive cell lines, blue; resistant cell lines, red. D, qPCR analysis of ID1 (left) and ID3 (right) gene expression after 1 hour of BMP4 treatment in five sensitive and five resistant lines. Expression levels are related to the untreated sample within each cell line. E, Knockdown of SMAD4 with siRNA in U3017MG cells decreased the BMP4-induced upregulation of ID1 and ID3 detected by qPCR analysis. siCtrl denotes the control siRNA. F, Fraction of EdU-positive cells in U3017MG pretreated with control siRNA (siCtrl) or siRNA against SMAD4 (siSMAD4) for 4 days and BMP4 the last 2 days. Six hours before fixation, EdU was added and the fraction of cells that had divided was determined for each condition. SMAD4 downregulation resulted in a significantly higher fraction of EdU-labeled cells than cells receiving control siRNA (two-way ANOVA, interaction P < 0.05). Error bars, SD based on four data points.

Figure 2. Canonical BMP4 signaling is functional in both resistant and sensitive cell lines, and is needed in a sensitive line to mediate BMP4 response. A, Overview of the BMP4 signaling pathway, highlighting the genes encoding the canonical signaling components. B, Scatter plots of baseline expression levels of BMP4 signaling components plotted against BMP4 resistance score. The resistant cell lines U3054MG (gray) and U3079MG (red), and the sensitive cell lines U3017MG and U3056MG (blue) are circled. C, Western blot analysis of phospho-SMAD1/5/9 activation in two sensitive and three resistant lines at 1 and 6 hours ± BMP4 (10 ng/mL). Total SMAD 1 and SMAD 5 levels are shown. Relative phospho-SMAD1/5/9 levels are depicted below the cyclophilin B loading control. The same blot was probed repeatedly with the antibodies used. Sensitive cell lines, blue; resistant cell lines, red. D, qPCR analysis of ID1 (left) and ID3 (right) gene expression after 1 hour of BMP4 treatment in five sensitive and five resistant lines. Expression levels are related to the untreated sample within each cell line. E, Knockdown of SMAD4 with siRNA in U3017MG cells decreased the BMP4-induced upregulation of ID1 and ID3 detected by qPCR analysis. siCtrl denotes the control siRNA. F, Fraction of EdU-positive cells in U3017MG pretreated with control siRNA (siCtrl) or siRNA against SMAD4 (siSMAD4) for 4 days and BMP4 the last 2 days. Six hours before fixation, EdU was added and the fraction of cells that had divided was determined for each condition. SMAD4 downregulation resulted in a significantly higher fraction of EdU-labeled cells than cells receiving control siRNA (two-way ANOVA, interaction P < 0.05). Error bars, SD based on four data points.

particles each. Thus, there is a potential for a wide spectrum of SOX2 expression levels in these cells, which were kept under constant selection to maintain the SOX2 vector. Notably, forced expression of SOX2 led to a significant downregulation of endogenous SOX2 protein, particularly in sensitive lines. Quantification of total SOX2 protein revealed that sensitive cells had basically the same total SOX2 protein levels upon transduction as cells harboring the control vector (−8% to +7%), while resistant cell lines could tolerate between 40% and 400% more than endogenous levels (Fig. 5B). This finding indicates that sensitive cells need to strictly control SOX2 protein levels to maintain a proliferative state.

Transduced cells were exposed to BMP4 and the cell density was determined at day 7 and 15. As shown in Fig. 5C, no effect on the proliferation of the three resistant lines was seen. However, in all sensitive lines, transduced SOX2 indeed conferred a partial resistance toward the growth-inhibitory activity of BMP4, indicating a causative role of SOX2 downregulation in BMP4-induced growth inhibition.

Inability to downregulate SOX2 transforms the BMP4 response pattern in a sensitive line toward a resistant-like pattern

Because the inability to downregulate SOX2 conferred a partial resistance on BMP4-sensitive cells lines, we wanted to identify transcriptional programs behind this effect. To this end, we utilized one sensitive (U3017MG) and one resistant (U3028MG) cell line transduced with the SOX2 vector or a control vector, and performed RNA sequencing on cells cultured with or without BMP4 for 14 days.
When analyzing BMP4-induced changes in gene expression, we could see a clear correlation in the resistant cell line (Spearman $r = 0.48$) compared with the sensitive cell line (Spearman $r = 0.23$) between cells harboring the SOX2 vector and control vector (Fig. 6B). This shows that constitutive SOX2 expression affects gene expression changes upon BMP4 exposure in the sensitive cell line to a larger extent than in the resistant cell line. In addition, multifactor analysis showed that the number of differentially expressed genes (significance level, $P < 0.0001$) and enriched GO gene sets (GSEA, nominal $P < 0.01$) were strikingly lower in resistant cells compared with the sensitive cells (Fig. 6C). Taken together, this demonstrates that constitutive SOX2 expression has a considerable effect on global gene expression changes upon BMP4 exposure in a sensitive line, while the effect in a resistant line is more modest.

Among the positively enriched BMP4-induced gene sets that were particularly upregulated in the sensitive cell line transduced with the control vector, we identified gene sets associated with extracellular matrix and stem cell division (Fig. 6D). In addition, genes associated with negative regulation of a MAPK-related signature...
Figure 4. BMP4-sensitive cell lines have high baseline SOX2 levels and downregulate SOX2 upon BMP4 exposure. 
A, Scatter plot of baseline SOX2 mRNA expression levels plotted against BMP4 resistance score. Pearson correlation coefficient = −0.524 with 95% confidence interval: −0.718−0.254. 
B, Western blot analysis of SOX2 protein in three sensitive and three resistant lines after 7 days ±BMP4 exposure. Relative SOX2 levels are depicted below the β-actin loading control.

Figure 5. Forced SOX2 expression downregulates endogenous SOX2 protein and renders sensitive cells more resistant to BMP4-mediated growth inhibition. 
A, Western blot analysis of three sensitive and three resistant cell lines with control vector (C) or SOX2 overexpression vector (S2). Myc-tagged SOX2 (top band) can be distinguished from endogenous SOX2 (bottom band) due to its size. Relative SOX2 levels are depicted below the β-actin loading control. 
B, Quantification of Western blot analysis in A. Numbers denote relative total SOX2 levels between control vector and SOX2 vector-expressing cells within the same cell line. 
C, Proliferation curves for GBM cell lines transduced with control vector (Ctrl-lv) or SOX2 overexpression vector (SOX2-lv) and cultured with or without BMP4 for 15 days. Top row, sensitive cell lines; bottom row, resistant cell lines. Error bars, SD based on four data points.
RNA sequencing of cells unable to downregulate SOX2 displays a transformation of the BMP4 response pattern toward a resistant-like pattern in a sensitive line. A, Schematic overview of samples analyzed in B–D. Cells were treated ±BMP4 for 14 days. B, Scatter plots showing log2 fold change of mRNA expression (BMP4-treated vs. untreated) in a sensitive cell line (U3017MG) and a resistant cell line (U3028MG) transduced with a SOX2 vector (SOX2-lv) or control vector (Ctrl-lv). Spearman correlation coefficients are indicated at the top right corners. C, Number of differentially expressed genes (P < 0.0001, left) and significantly changed GO gene sets (P < 0.01, right) in U3017MG and U3028MG with SOX2 vector ±BMP4 or control vector ±BMP4. D, Top 6 enriched GSEA GO signatures differing between U3017MG and U3028MG with SOX2 vector ±BMP4 or control vector ±BMP4. E, Schematic overview of cells included in the multifactor analysis in F. Cells were treated ±BMP4 for 14 days. F, Number of differentially expressed genes (P < 0.001) between two sensitive cell lines ±BMP4 (U3017MG and U3065MG) and two resistant cell lines ±BMP4 (U3019MG and U3028MG; left circles) and the overlap with the differentially expressed genes between U3017MG with SOX2 vector or control vector ±BMP4 (top right circle), or U3028MG with SOX2 vector or control vector ±BMP4 (bottom right circles).
were found among the positively enriched gene sets (GO_NEGATIVE_REGULATION_OF_ERK1_AND_ERK2_CASCADE, P = 0.006, NES = 1.60). This provides cues to functional processes where BMP4-mediated upregulation is dampened more in a sensitive line than in a resistant one when SOX2 is constitutively expressed.

Finally, we investigated whether inability to downregulate SOX2 in response to BMP4 makes U3017MG behave like a resistant cell line. To this end, we examined the BMP4-induced differentially expressed genes (P < 0.001) between sensitive and resistant cell lines that overlap with the BMP4-induced differentially expressed genes between cells harboring control vector or SOX2 vector (both U3017MG and U3028MG; Fig. 6E). A significant number of genes (524) overlapped with U3017MG (χ² P < 0.0001) while the same trend could not be seen for U3028MG (χ² P = 0.013; Fig. 6F). This demonstrates that the BMP4 response in U3017MG is changed toward a resistant response pattern upon constitutive SOX2 expression.

Among the 524 common BMP4-induced genes in sensitive cell lines and U3017MG harboring the control vector, a considerable number of histone proteins could be found, representing gene sets related to broad epigenetic changes (Supplementary Table S1). The list of positively enriched GO terms also includes gene sets associated with MAPK signaling and, in particular, its negative regulation (MAPK cascade, P = 0.00016; negative regulation of MAP kinase, P = 0.0048; and inactivation of MAPK activity, P = 0.012). This finding suggests that the ability to upregulate genes that inhibit MAPK signaling is one of the main functions dampened by constitutive SOX2 expression in a BMP4-sensitive cell line.

**Discussion**

Previous reports on the growth-inhibitory effect of BMP4 on human GBM cells have provided conflicting results. The view of BMP4 as an effective and irreversible growth inhibitor and antitumor agent presented by Piccirillo and colleagues (7) was refuted by Carén and colleagues (8), who found that only a subset of GBM cell cultures responded to BMP. In this study, we could identify a few cell lines which responded to BMP4 with an almost total cessation of proliferation, while other cell lines were completely refractory, with a range of responsiveness between the extremes. Thus, our results provide a clear illustration of the heterogeneity of GBM, which is a hallmark of this tumor type. We proceeded to search for an mRNA expression profile corresponding to the BMP4 sensitivity, and identified a putative stem cell profile and a SOX2-dependency coupled to sensitivity.

BMPs exert their actions through binding and activation of heterotetrameric complexes of type I and type 2 BMP receptors (20). Receptor activation elicits an intracellular cascade of canonical signaling thorough pSMAD1,5,9/SMAD4 complexes, as well as activation of a noncanonical signaling pathway. Even though we could show that the inhibitory activity of BMP4 in sensitive lines is dependent on canonical BMP4 signaling, we found no evidence that resistance was associated with silencing of this pathway. Lee and colleagues (28) previously demonstrated that epigenetic silencing of BMPR1B inhibited BMP2-induced differentiation in a single line of GBM cells. Although we cannot exclude the possibility that the expression levels of BMP receptors participate in the fine-tuning of BMP response, our results demonstrate that this is not a general or major determinant.

Previous studies have shown that GBM cells of the mesenchymal phenotype are less sensitive to drugs and radiation (29–31). We observed a connection between BMP4 sensitivity and the stem cell-like pronuclear subtype. SOX2 is a well-established stem cell transcription factor needed to maintain the proliferative capacity of glioblastoma cells (25, 26, 32). BMP2 and BMP4 have been demonstrated to downregulate SOX2 (27, 28, 33, 34), as confirmed by our work. Interestingly, this downregulation was most apparent in sensitive lines; one of the resistant lines had but a modest downregulation, while another had remarkably low basal levels of SOX2. Strikingly, all tested resistant cell lines displayed higher total levels of SOX2 protein upon ectopic expression of SOX2, while the SOX2 levels in sensitive lines remained unchanged, with an almost complete downregulation of the endogenous SOX2 protein. Clearly, the well-established negative feedback regulation of SOX2 expression is operational in the BMP4-sensitive cells, but may be out of order in the resistant cells. SOX2 levels can be controlled transcriptionally and posttranscriptionally, where the latter has been shown to play a role in embryonic stem cell pluripotency (35) and neural progenitor cell differentiation (36). In our work, lentiviral SOX2 expression provides a source that cannot be transcriptionally regulated, but posttranscriptional regulation of ectopic SOX2 may still be in action. In cortical progenitor cells, high SOX2 levels maintain stem cells in a slow cycling state (37) and in GBM, acute SOX2 overexpression can render a complete stop in proliferation (38). We therefore consider it likely that transduced cells expressing very high levels of SOX2 are selected against, thus explaining the fact that the basal level of SOX2 was maintained. Together with our data, this points toward a requirement of preserving precise SOX2 levels in BMP4-sensitive GBM cell lines, where neither too high nor too low levels are tolerated. In support of this conception, we found that BMP4-sensitive GBM lines expressing ectopic SOX2 were partially refractory to BMP4. Resistant cell lines, however, appear to tolerate a broader spectrum of SOX2 levels and remain proliferative. This was seen in U3028MG where BMP4-mediated downregulation of SOX2 does not induce growth arrest, and in all three tested resistant cell lines expressing ectopic SOX2 at higher levels than endogenously found in these cells.

Our results suggest that a SOX2-dependent upregulation of genes involved in negative regulation of the MAPK pathway is a key component in BMP4-mediated growth inhibition. Apparently, this mechanism is out of order in resistant cell lines. Along the same line, Riddick and colleagues (39) linked MAPK activation to a regulatory circuit involving SOX2 in GBM cells.

As suggested by Piccirillo and colleagues (7), harnessing the BMP4 growth-inhibitory pathway may be a fruitful approach in the search for a more effective treatment of GBM. Studies on the effect of local BMP administration to experimental GBM in mice support this view (40, 41). Positive experimental data on the inhibitory effect of BMP on GBM cells have been an incentive to initiate a phase I clinical trial on BMP4 treatment in recurrent GBM (42). However, our present results and findings by Carén and colleagues (8) show that only a subset of GBM cell lines may be effectively growth inhibited by BMP4. On the basis of the present findings, we propose that a SOX2-regulated program connected to stemness is a critical determinant for BMP4-induced growth retardation in GBM cells. Further studies on SOX2 and other biomarkers predicting BMP4 response may be important for the identification of patients which will benefit from BMP4 treatment in future clinical studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: E. Dalmo, B. Westermark

Development of methodology: S. Nelander, B. Westermark

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Dalmo, B. Westermark

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Dalmo, P. Johansson, I. Gustavsson, S. Nelander, B. Westermark

Writing, review, and/or revision of the manuscript: E. Dalmo, P. Johansson, M. Niklasson, I. Gustavsson, S. Nelander, B. Westermark

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Dalmo, I. Gustavsson

Study supervision: S. Nelander, B. Westermark

Other (performed experimental work): M. Niklasson

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Erika Dalmo, Patrik Johansson, Mia Niklasson, et al.


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