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Bone morphogenetic protein receptor inhibitors suppress the growth of glioblastoma cells

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Abstract

Glioblastomas (GBMs) are aggressive brain tumors that are resistant to chemotherapy and radiation. Bone morphogenetic protein (BMP) ligand BMP4 is being examine as a potential therapeutic for GBMs because it induces differentiation of cancer stem cells (CSC) to an astrocyte phenotype. ID1 is reported to promote self-renewal and inhibit CSC differentiation. In most cancers, ID1 is transcriptionally upregulated by BMP4 promoting invasion and stemness. This conflicting data brings into question whether BMP signaling is growth suppressive or growth promoting in GBMs. We utilized BMP inhibitors DMH1, JL5, and Ym155 to examine the role of BMP signaling on the growth of GBMs. DMH1 targets BMP type 1 receptors whereas JL5 inhibits both the type 1 and type 2 BMP receptors. Ym155 does not bind the BMP receptors but rather inhibits BMP signaling by inducing the degradation of BMPR2. We show that JL5, DMH1, and Ym155 decreased the expression of ID1 in SD2 and U87 cells. JL5 and Ym155 also decreased the expression of BMPR2 and its down-stream target inhibitor of apoptosis protein XIAP. JL5 treatment resulted in significant cell death and suppressed self-renewal to a greater extent than that induced by BMP4 ligand. The lysosome inhibitor chloroquine increases the localization of BMPR2 to the plasma membrane enhancing JL5 induced downregulation of ID1 and cell death in SD2 cells. We show that BMP signaling is growth promoting in GBMs. These studies suggest the need for development of BMP inhibitors and evaluation as potential therapeutic for GBMs.

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Authors' Contributions

JK contributed to data acquisition and figure preparation, and co-wrote the method section, AM contributed to acquisition of data, co-wrote the methods section, and edited drafted manuscript. RF, critically reviewed and edited the manuscript, DJ contributed to data acquisition, JL contributed to study design, preparation of figures, and wrote the manuscript. All authors approved the manuscript.

Conflict of interests:

The authors declare that they have no conflict of interest.

Institutional Approvals

The use of BMP inhibitors and cell lines have been approved by Rutgers University Institutional Biosafety Committee #13-424.

Keywords

BMP; BMPR2; BMP inhibitors; Smad-independent; glioblastoma; cancer stem cells; ID1; cell survival; self-renewal

Introduction

Glioblastomas are highly aggressive brain tumors effecting approximately 12,000 people per year in the United States [1]. Brain tumors are the 3rd leading cause of cancer deaths between the ages of 15–39. Glioblastomas are universally fatal with mean survival of 14 months [2]. Glioblastomas are treated with the combination of radiation, chemotherapy, and surgery [2]. These dismal results make it clear new cancer therapeutics are needed for the treatment of malignant brain tumors.

Cancer cells within glioblastomas have characteristics similar to those of neural stem cells. Cancer stem cells (CSC) are tumor-initiating cells that self-renew and differentiate [3]. The glial cancer stem cell population is also resistant to chemotherapy and radiation [4]. The bone morphogenetic proteins (BMPs) are known to induce the differentiation of normal neural stem cells into astrocytes [5]. Studies have revealed that BMP ligands have a growth suppressive effect on glioblastoma CSCs [6–8]. The mechanisms by which BMP ligands suppress growth of glioblastomas are not fully elucidated. However, studies suggest that BMP4 may be mediated by inducing the differentiation of CSCs into astrocytes [6, 9].

BMPs are transcription activators of inhibitor of differentiation proteins, ID1–4. BMP ligands, BMP4 and BMP2, bind to type 1 BMP receptors (BMPR1), which are then activated by the BMP type 2 (BMPR2) receptors [10]. The BMPR1/BMPR2 complex then phosphorylates Smad-1/5 factor promoting its translocation to the nucleus inducing the transcription of ID1–4 [11–13]. BMP2/4 are potent activators of ID1 in many cancers including glioblastoma CSCs [14–16]. ID1 regulates cell invasion, proliferation, and self-renewal in many cancers including glioblastoma CSCs [14, 17–24].

BMP4 ligand is currently being evaluated as a potential therapy for glioblastomas. Interestingly, BMP4 has also been shown to induce quiescence of CSCs leading to chemotherapy resistance through its upregulation of ID1 [16]. How BMP4 suppresses glioblastomas while increasing ID1 expression, which is reported to enhance tumorigenesis, is not understood. BMP receptor inhibitors have been shown to inhibit growth of other cancer cell types through a process, which involves the downregulation of ID1 [14, 25, 26]. Gain of function mutations of alk2 occur in 25% of the rare pediatric brainstem tumor diffuse high-grade astrocytoma [27]. Gain of function mutations of alk2 occur in 95% of patients with Fibrodysplasia ossificans progressive (FOP), which is characterized by ectopic ossification of soft tissues [28, 29]. Laboratories have developed specific inhibitors of the type I BMP receptors to treat diseases with overactive BMP signaling. BMP inhibitors are expected to enter clinical trials shortly [30, 31]. BMP receptor inhibitors have not been evaluated in glioblastomas.

We hypothesized that inhibition of BMP receptor signaling will decrease ID1 expression and suppress growth of glioblastomas. Using BMP inhibitors, we show that following suppression of BMP signaling there is a significant decrease in the expression of ID1. BMP inhibitors significantly suppressed growth and self-renewal of glioblastoma cell lines compared to BMP4. These studies suggest that suppressing BMP signaling rather than inducing its activation may be a better strategy to suppress growth of glioblastomas.

Material and Methods

Cell culture and reagents

SD2 cells were derived from a primary glioblastoma that have been maintained in neural stem cell medium (Neurocult NS-A proliferation medium, Stemcell Technologies, WA, USA). SD2 cells were obtained from Roland H. Friedel (Department of Neurosurgery, Icahn School of Medicine at Mount Sinai, New York, NY) who authenticated and characterized the cell line [32]. The U87 MG cell line was purchased from ATCC and characterized genetically at COSMIC cancer database. U87 MG cells were cultured in EMEM medium (Sigma Aldrich, USA) supplemented with 10% FBS. For SD2 cells, plates were coated with 10ug/ml laminin (Sigma Aldrich, USA) for 4 hours at 37°. GBM2 and GBM3 are human primary undifferentiated grade IV glioblastoma cell lines obtained from Ramsey Foty [33, 34] (Department of Surgery, Rutgers Robert Wood Johnson Medical School). These cell lines were maintained in Eagles' Minimal Essential Medium (EMEM)/10% fetal calf serum and antibiotics. Cell culture experiments performed in a mycoplasma free environment. JL5 was synthesized by Dr. Jacques Roberge and John Gilleran at Rutgers University, Molecular Design & Synthesis [35]. YM155 was purchased from Selleckchem (Houston, TX). Chloroquine and BMP4 were purchased from Sigma Aldrich, USA and R&D systems, USA, respectively.

Cell viability

For SD2 cell, 300,000 cells/well and for U87 cell 400,000 cells/well were seeded in duplicate into 6-well plates and grown overnight at 37° incubator with 5% CO2. The cells were then treated for the designated time period. After the treatment, SD2 cells were harvested with accutase and U87 cells were harvested with 0.05% trypsin. An automated Vi-CELL cell analyzer (Beckman Coulter, USA) was used to determine the percent dead cells and total number of live cells in each treatment group. Each experiment was replicated at least 4 times in our laboratory.

Immunofluorescence staining

SD2 and U87 cells were seeded overnight onto microscope cover glasses in a 6 well plate. Cells were treated with 2.5uM JL5, 20nM Ym155 and 80uM Chloroquine for designated period. Cells were then washed with PBS and fixed 4% formaldehyde. Permeabilized was performed with 0.5% triton-X. To determine BMPR2 on the plasma membrane, the permeabilization step was not performed. The cells were blocked with CAS-block (Life Technologies, USA) for 1h and then incubated with anti-BMPR2 antibody (Sigma-Aldrich, USA) for 1h at room temperature. This antibody recognizes an extracellular epitope of BMPR2. Then the cells were washed with PBS and incubated with Alexa Flour 488

conjugated secondary antibody at 1:100 dilution for 1h at room temperature. After washing with PBS, the nuclei were counterstained with DAPI (Sigma-Aldrich, USA) for 10 min. Fluorescent images were captured using an Nikon eclipse TE300 inverted epifluorescent microscope (60X oil lens) and a Cool Snap black and white digital camera. IP Lab imaging software was used to assign pseudo-color to each channel. ImageJ (NIH, USA) software was used to determine the mean fluorescence intensity and count BMPR2 positive cells.

Western blot analysis

Cells were seeded overnight in 6 well plates and then treated for the designated period. Total cellular protein was extracted using RIPA buffer and the protein concentration was determined by using Pierce BCA protein assay kit (ThermoFisher, USA). Equal amounts of proteins were loaded onto a polyacrylamide gel and separated by SDS-PAGE. The proteins were transferred overnight to a nitrocellulose membrane (Biorad, USA). The membranes were blocked for 1h using membrane blocking solution (Life Technologies, USA) and then incubated overnight with primary antibody at 4°C. The primary antibodies used were rabbit monoclonal anti-Id1 (Calbioreagents, San Mateo, CA), rabbit monoclonal XIAP, rabbit monoclonal beta III tubulin (Tuj1), rabbit monoclonal anti-pSmad 1/5 (Cell Signaling Technology, MA, USA), rabbit polyclonal anti-Smad 1/5 (Upstate Biotechnology, NY, USA), rabbit anti-actin, rabbit polyclonal anti-GAPDH (Sigma, St. Louis, MO), and mouse monoclonal anti-spectrin (EMD Millipore, CA, USA). Rabbit polyclonal SOX2 (Abcam, USA), mouse monoclonal anti-NeuN (Chemicon, USA), and mouse monoclonal anti-Nestin (Chemicon, USA).

3D-Sphere forming ability

To evaluate 3D-sphere forming ability, 1000 cells were plated per will in 12 well plates. To facilitate the sphere formation, the plate was not coated with laminin. Next, the cells were treated with DMSO, 40ng/ml BMP4 and 2.5uM of JL5 for 7 days then 4 20x images were taken per well using a Nikon eclipse TE300. Using ImageJ (NIH, USA) software the total number of spheres was counted. A histogram was plotted using average number of spheres in each treatment group.

Extraction of membrane proteins:

The membrane proteins were extracted using Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Scientific #89842) according to the manufacturer's protocol. Briefly, 1×10^6 cells were grown overnight and then treated for 24 hours. After treatment, cells were collected by scraping, and then cell suspension was centrifuged at 300 × g for 5 minutes. The cell pellet was washed twice with Cell wash solution and centrifuged at 300 × g for 5 minutes. 100 μl of Permeabilization Buffer was added to the cell pellet and incubated for 10 minutes at 4°C. The permeabilized cells were then centrifuged at 16,000 × g for 15 minutes. The supernatant was collected as cytosolic proteins. Solubilization Buffer was added to the cell pellet and incubated for 30 minutes at 4°C. Solubilized membrane and membrane-associated proteins were collected after centrifuging the tubes 16,000 × g for 15 minutes at 4°C.

Statistical Analysis

The mean of the control group was compared to the mean of each treated group using a paired student t-test assuming unequal variances. Differences with p values <.05 were considered statistically significant.

RESULTS

JL5 treatment suppresses growth and downregulates ID1 expression of glioblastoma cells

JL5 is a potent inhibitor of the BMP type 1 receptors alk2, alk3, and alk6 [35]. JL5 has some inhibition for BMPR2 but also suppresses BMPR2 signaling by causing its mislocalization to the cytoplasm [35, 36]. We have previously reported that JL5 is a more potent inhibitor of BMP signaling and growth suppression of lung cancer cells than inhibitors specific for BMPR1 (DMH1) [19, 35, 36]. The greater suppression of BMP signaling induced by JL5 is thought to be due to its ability to suppress BMPR2 smad-independent signaling. Here we show that JL5 treatment resulted in significant cell death and growth suppression of SD2 cells (Figure 1a–b). However, JL5 treatment of U87 cells, which are in a more differentiated state than SD2 cells, did not result in significant cell death, but appeared to significantly suppress growth (Figure 1d–e). JL5 significantly decreased the expression of ID1 in both SD2 and U87 cells (Figure 1c, f).

Effects of DMH1 on cell growth and ID1 expression.

DMH1 is a selective inhibitor of the BMP type 1 receptors (alk2, alk3, and alk6) with no activity for BMPR2 [37]. In SD2 cells, DMH1 caused less growth suppression, cell death, and downregulation of Id1 than JL5 (Figure 2a–b). DMH1 also decreased ID1 expression in SD2 cells (Figure 2c). In U87 cells, DMH1 did suppress growth and caused a significant decrease in the expression of ID1 (Figure 2c–f).

Effects of JL5 and DMH1 on GBM2 and GBM3 cell lines

To further examine the effects of JL5 compared to DMH1, primary human glioblastoma cells lines GBM2 and GBM3 were treated with each compound at the same time. Neither of the compounds induced cell death (Figure 3a,d). JL5 caused significant growth suppression and downregulation of the expression of ID1 in both GBM2 and GBM3 cell lines (Figure 3). DMH1 had no significant effect on cell growth (Figure 3 b,e). Although DMH1 did suppress ID1 expression in both cell lines it was less compared to that of JL5 (Figure 3 c,f). Together, these studies show that BMP receptor small molecule inhibitors decrease ID1 expression suggesting BMP is an upstream regulator of ID1. Growth inhibition correlated with the degree of ID1 downregulation.

Regulation of BMP4 on ID1 expression and cell growth

Next, we used BMP4 ligand to assess if BMP signaling is an upstream regulator of ID1 and exam ligands effects on cell growth. In SD2 cells, BMP4 activated Smad-1/5 and increased the expression of ID1 within 1 hour, and persisted for at least 5 days (Figure 4a). After 5 days of treating SD2 cells with BMP4 approximately 13% of the cells were dead (Figure 4b). In comparison, 50% of SD2 cells were dead after 5 days of treatment with JL5 (Figure

1a). The number of live cells was also decreased after 5 days but the change was not as significant as seen with JL5 (Figure 4b). In U87 cells, BMP4 also caused an increase in the activation of pSmad-1/5 and ID1 expression at 1 hour and 5 days but the increase was not as robust as that observed for SD2 cells (Figure 4c). However, the baseline phosphorylation of Smad-1/5 was higher in the U87 cells. Treating U87 cells with BMP4 for 5 days caused a significant increase in cell growth (Figure 4d).

Ym155 decreases ID1 and XIAP expression and induces cell death

Ym155 was originally reported to be a survivin inhibitor [38]. We found that Ym155 potently decreases BMP signaling and induces cell death of non-small cell lung carcinomas (NSCLC) cell lines [39]. Ym155 inhibition of BMP signaling is mediated by causing the mislocalization of BMPR2 to the cytoplasm and enhancing its degradation [39]. In both SD2 and U87 cells, Ym155 treatment results in significant cell death with few remaining live cells after 5 days (Figure 5a, c). In both cells lines, Ym155 decreased the expression of ID1 (Figure 5b, d). In SD2 but not in U87 cells, Ym155 decreased ID1 expression to a greater extent than JL5 (Figure 5b, d). Ym155 decreased the expression of BMPR2 and its' down-stream target XIAP in SD2 cells similar to that of JL5 (Figure 5e). Neither Ym155 nor JL5 decreased the expression of BMPR2 or XIAP in U87 cells (Figure 5f). Inhibition of BMP type I receptors does not down-regulate XIAP in lung cancer cells [19, 36]. DMH1 also did not down-regulate XIAP in SD2 cells (Figure 5g). These studies suggest that Ym155 and JL5 but not DMH1 down-regulates BMPR2 Smad-independent signaling in SD2 cells.

Ym155 and JL5 do not regulate differentiation

BMP4 signaling not only induces differentiation of NSC into astrocytes, it inhibits their differentiation into neurons [40]. We asked whether the suppression of BMP signaling with JL5 or Ym155 would promote neuronal differentiation in glioblastomas. SD2 cells have a higher expression of the NSC markers nestin and Sox2 in comparison to the U87 cells (Figure S1). SD2 cells also have a higher expression of the neuronal markers Tuj1 and NeuN in comparison to U87 cells (Figure S1). Other than a decrease in Sox2 in SD2 induced by Ym155, there was no significant change in any of the markers by Ym155 or JL5 in either the SD2 or U87 cells (Figure S1). Based on this study, we see no evidence that inhibition of BMP signaling promotes neuronal differentiation in glioblastomas.

Chloroquine increases BMPR2 cell membrane expression and enhances JL5 induced cell death

We previously reported in NSCLC cell lines that JL5 and Ym155 cause the mislocalization of BMPR2 to the cytoplasm with a corresponding decrease in the BMPR2 expressed on the plasma membrane [39]. Using an antibody recognizing the external epitope of BMPR2, we found that there was significantly fewer U87 cells expressed BMPR2 in comparison to SD2 cells (Figure 6a). Both JL5 and Ym155 decreased the expression of BMPR2 in SD2 cells (Figure 6b).

Chloroquine decreases the lysosomal degradation of BMPR2 resulting in an increase in BMPR2 being expressed on the cell surface [41]. Chloroquine significantly increased the

intensity and the number of cells expressing BMPR2 in both the SD2 and U87 cells (Figure 6c,e). Chloroquine also increased BMPR2 protein expression in the membrane fraction while decreasing expression in the cytosol (Figure 6d). Chloroquine also increased the expression of ID1 demonstrating an increase in BMP signaling (Figure 6f). SD2 cells pretreated with chloroquine were more responsive to JL5 as demonstrated by a greater decrease in the expression of ID1 (Figure 6g). The combination of JL5 with chloroquine increased the downregulation of Id1 and significantly enhanced cell death of SD2 cells compared to either compound alone (Figure 6h).

BMP inhibitors decrease self-renewal

The ability to form spheres from single cells is commonly used to assess self-renewal of glioblastomas CSC populations. Similar to what has been previously reported, BMP4 ligand suppressed self-renewal of glioblastoma cells (Figure 7). Spheres formed following treatment with BMP4 were smaller and less in number (Figure 7). JL5 completely prevented sphere formation of glioblastoma cells (Figure 7). These data suggest that inhibition BMP signaling suppresses self-renewal more than its activation.

Discussion

Terminally differentiating glial cancer stem cells into non-cycling astrocytes with BMP4 is a novel and intriguing therapeutic approach for glioblastoma. Initial studies showed that BMP4 reduced the number of glial tumor-initiating cells and effectively blocked tumor growth in mice after intracerebral grafting [6]. Subsequent studies have revealed challenges with BMP4 directed therapy [42]. Many patient-derived glioblastomas are resistant to the growth suppressive effects of BMP4 [43]. Of the glioblastomas that are responsive to BMP4, DNA methylation was incomplete and cells could re-enter the cell cycle when challenged with growth factors [44]. Activation of BMP signaling has also been shown to induce quiescence of glial cancer stem cells, which are resistant to radiation and temozolomide chemotherapy [16]. Other challenges include the ability to deliver BMP4 across the blood-brain barrier.

Adding to the complexity of BMP signaling in glioblastomas is its role in the regulation of ID1. ID1 enhances the tumorigenicity of several cancers including glioblastomas. Knockout of ID1 in glioblastomas decreased invasiveness, prevented tumor progression, and inhibited glial cancer stem cell self-renewal [45–47]. One study suggested that ID1 promoted self-renewal of glial cancer stem cells by suppressing the expression of BMPR2 [24]. In this paradigm, ID1 is an upstream regulator of BMP signaling. Multiple studies have shown that BMP signaling is an upstream transcriptional regulator of ID1 [11–13]. ID1 has been shown to be a direct target of BMP ligands in normal, cancer, and embryonic stem cells [15] [26] [11]. In our studies, suppression of BMP signaling with inhibitors significantly decreased ID1 expression without increasing the expression of BMPR2. In the present study, BMP ligands potently increased the expression of ID1 in glioblastoma cell lines indicating ID1 is a downstream target of BMP signaling. We conclude that BMP signaling is upstream of ID1 in glioblastomas and its suppression it likely contributing to the growth suppressive effects observed with BMP inhibition.

Glioblastoma consist of heterogenous cell populations consisting of cells with stem cell-like (CSC) properties as well are cells that are more differentiated [48]. CSC are tumor promoting and are more resistant to chemotherapy and radiation. Eradication of CSC are thought to be required to eliminate tumor burden. Non-CSC can acquire stem cell like properties depending on the microenvironment or in response to therapy [48]. ID1 promotes self-renewal of embryonic and hematopoietic stem cells [49] as well as CSC population in glioblastomas and other cancers [47, 50]. ID1 also regulates proliferation and invasiveness of cancer cells. Knockdown of ID1 in unselected cancer cell lines diminishes invasiveness and proliferation [45, 46]. These studies suggests that ID1 cancer promoting properties are not isolated only to the CSC population.

Our studies suggest that the inhibition of BMP signaling rather than its stimulation will have a greater effect suppressing growth and inducing cell death of glioblastoma, including its' cancer stem cell population. Our studies have suggested that suppression of BMPR2 causes more cell death in cancer cells than the inhibition of BMP type 1 receptors [36]. BMP signaling has been shown in other cancers to stimulate pathways known to enhance survival. Some of these cell survival pathway are mediated by BMPR2 independent of BMPR1 and the activation of Smad-1/5. BMPR2 regulated pathways include the potent ant-apoptotic proteins XIAP and TAK1 [19, 51, 52]. One study suggested that inhibition of BMP type 1 receptor decreased oncogenic features of malignant astrocytes. Transgenic deletion of BMP 1A receptor (alk3) in astrocyte transformed with oncogenic Ras and deletion of p53 decreased oncogenic astrocytes proliferation, decreased migration, and decreased invasion [53]. Authors also report that DMH1 suppressed proliferation of oncogenic astrocytes more than BMP4 [53]. We cannot exclude that the cell death induced by JL5 was not mediated in part by other mechanism such as suppression of TGFß signaling. More potent and specific BMPR2 inhibitors are needed to address these important questions.

BMPR2 is cycled from the plasma membrane to the cytosol and back to the plasma membrane by recycling endosomes [54]. BMPR2 can also be trafficked to the lysosome for proteolytic degradation. Chloroquine increases lysosomal pH, which inhibits the degradation of cell surface protein including BMPR2 [41]. Chloroquine caused an increase expression of BMPR2 in SD2 and U87 cells with an increase in expression of ID1. Surprisingly, Chloroquine together with JL5 significantly enhanced cell death. These data suggest that the synergistic cell death induced by co-treatment was because the cells were more sensitive to BMP inhibition as a result of BMPR2 expression.

Conclusion

The studies presented here suggest that inhibition of BMP signaling is a potential therapeutic approach to treat glioblastomas. Our preliminary observations also suggest that synergistic cell death occurs with JL5 and chloroquine. Further investigation is needed with more specific BMP inhibitors in a larger number of glioblastomas both in vitro and in vivo tumor models to validate these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on reasonable request

Abbreviations

GBMs	glioblastomas
CSC	cancer stem cells
BMPs	bone morphogenetic proteins
BMPR	bone morphogenetic protein receptor
ID1	inhibitor of differentiation protein 1
CO2	carbon dioxide
PBS	phosphate buffered saline
NIH	National Institute of Health
XIAP	X-linked inhibitor of apoptosis protein
TAK1	transforming growth factor-β-activated kinase 1

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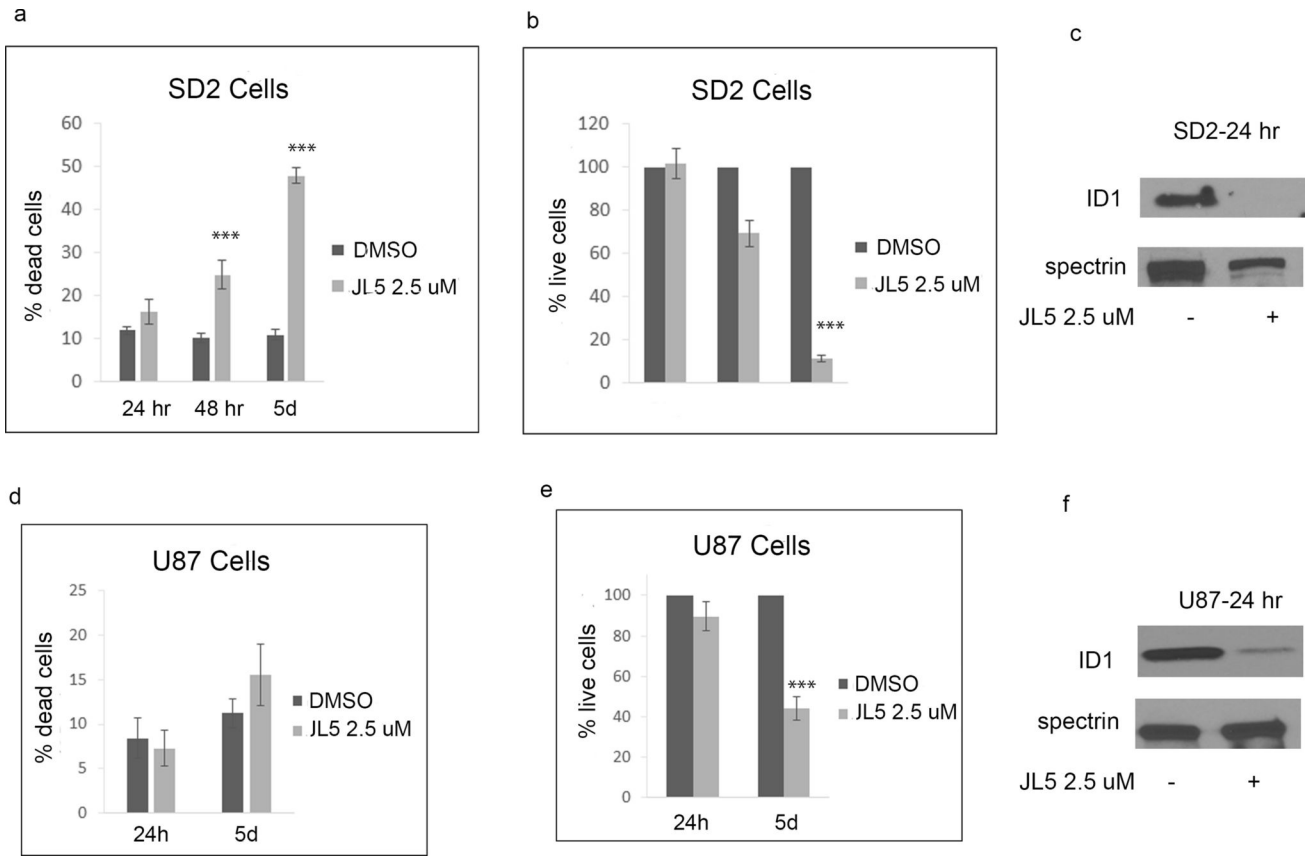

Figure 1. JL5 suppresses growth of glioblastoma cell lines and down-regulates the expression of ID1.

(a,b,d,e) SD2 and U87 cells were treated with JL5 for 5 days and the percentage of dead cells and number of live cells determined. Data represents the mean of 4 independent experiments. (c,f) Western blot analysis of SD2 and U87 cells treated with JL5 demonstrating a decrease in ID1 expression. *** $p < 0.0005$ compared to control.

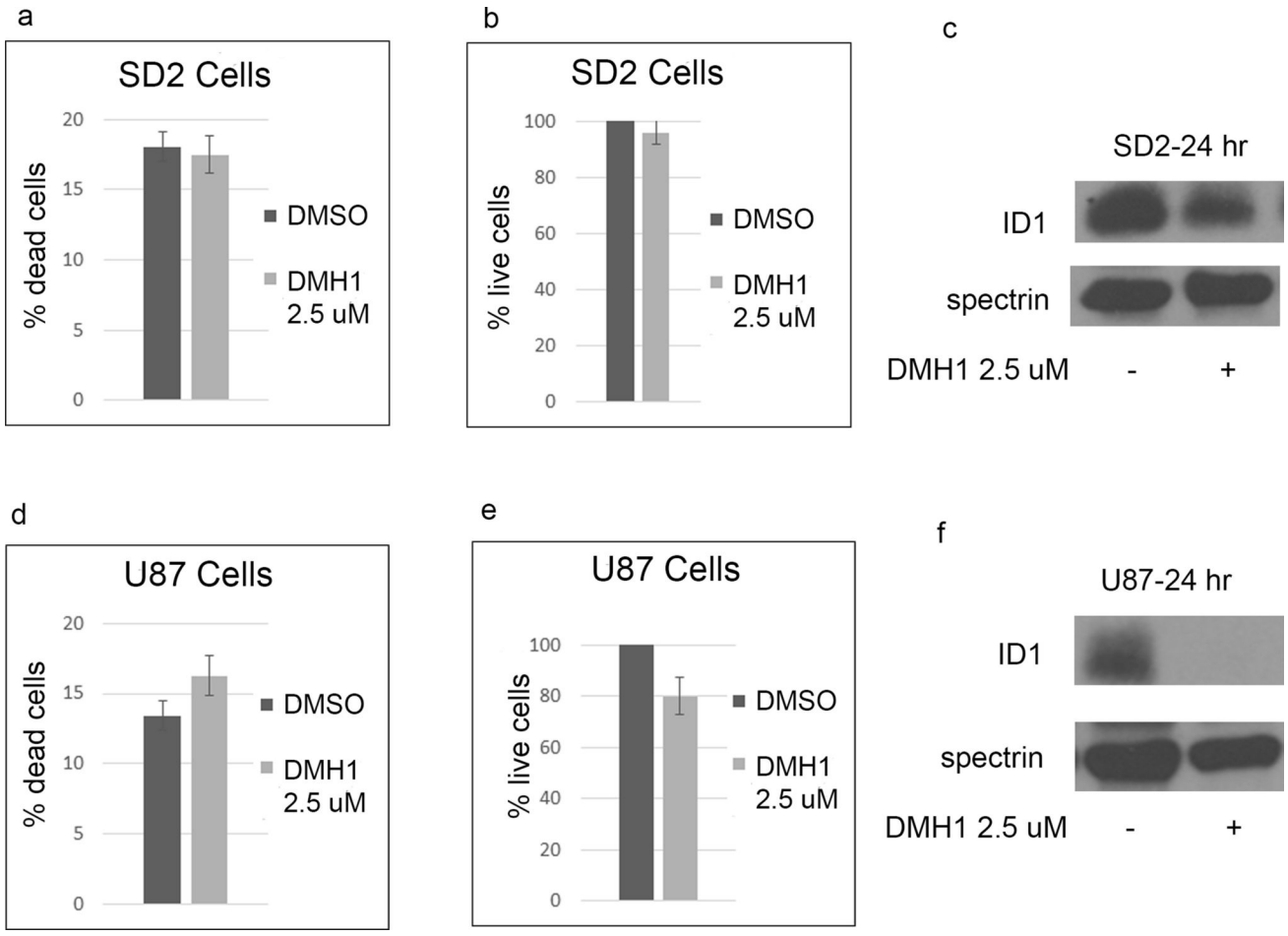

Figure 2. Inhibitor of BMP type 1 receptor DMH1 decreases ID1 expression with little effect on cell survival

(a,b,d,e) SD2 and U87 cells were treated with DMH1 for 5 days and the percentage of dead cells and number of live cells determined. Data represents the mean of 4 independent experiments. (c,f) Western blot analysis of SD2 and U87 cells treated with DMH1 demonstrating a decrease in ID1 expression, which was more in U87 cells.

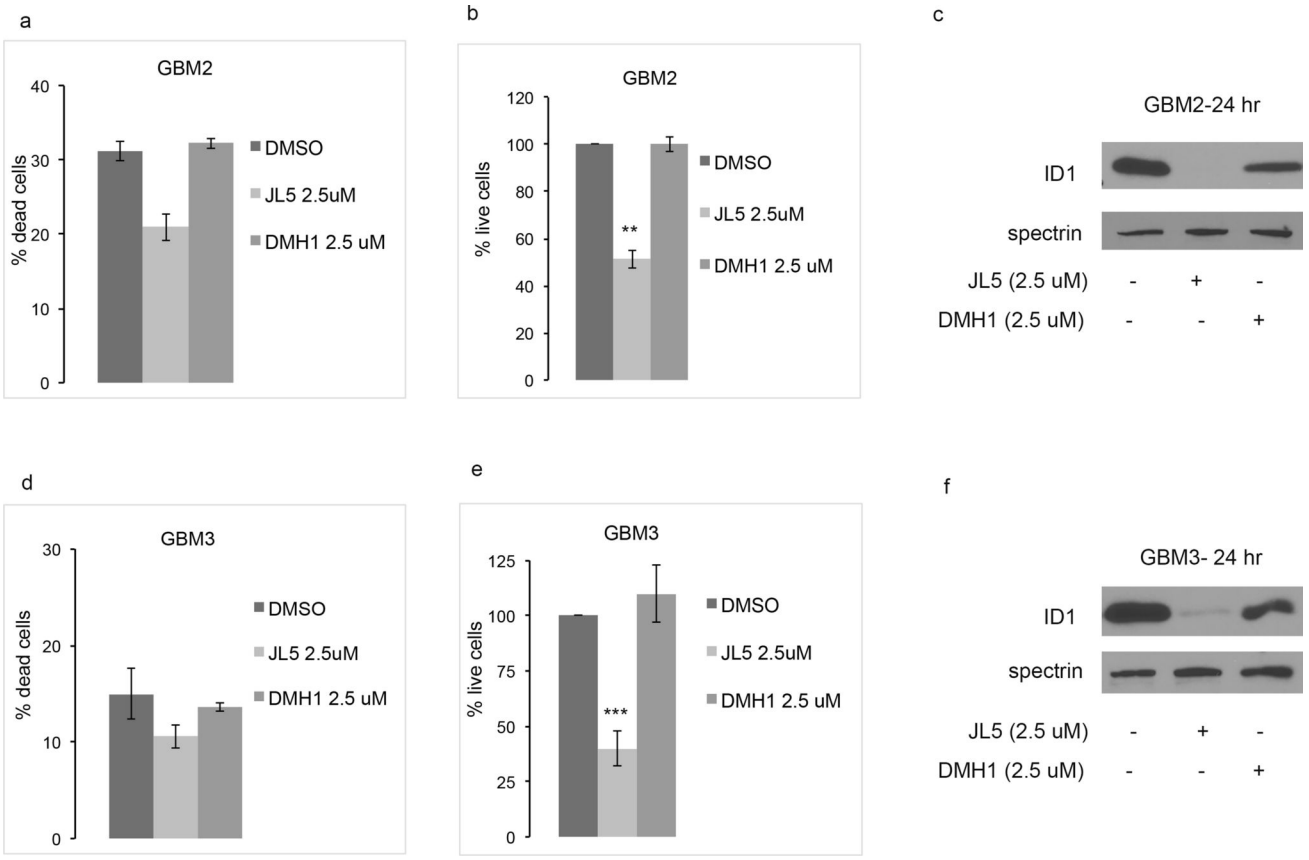

Figure 3.

JL5 decrease cell growth and ID1 expression in primary glioblastomas GBM2 and GBM3. (a,b,d,e) GBM2 and GBM2 glioblastoma cell lines were treated with JL5 or DMH1 for 5 days and the percentage of dead and live cells determined. Data represents the mean of 4 experiments for GBM3 and 2 experiments for GBM2. (C,F) Western blot analysis of GBMs treated with JL5 or DMH1 for 24 hr. **p<0.005, *** p <0.0005 compared to control.

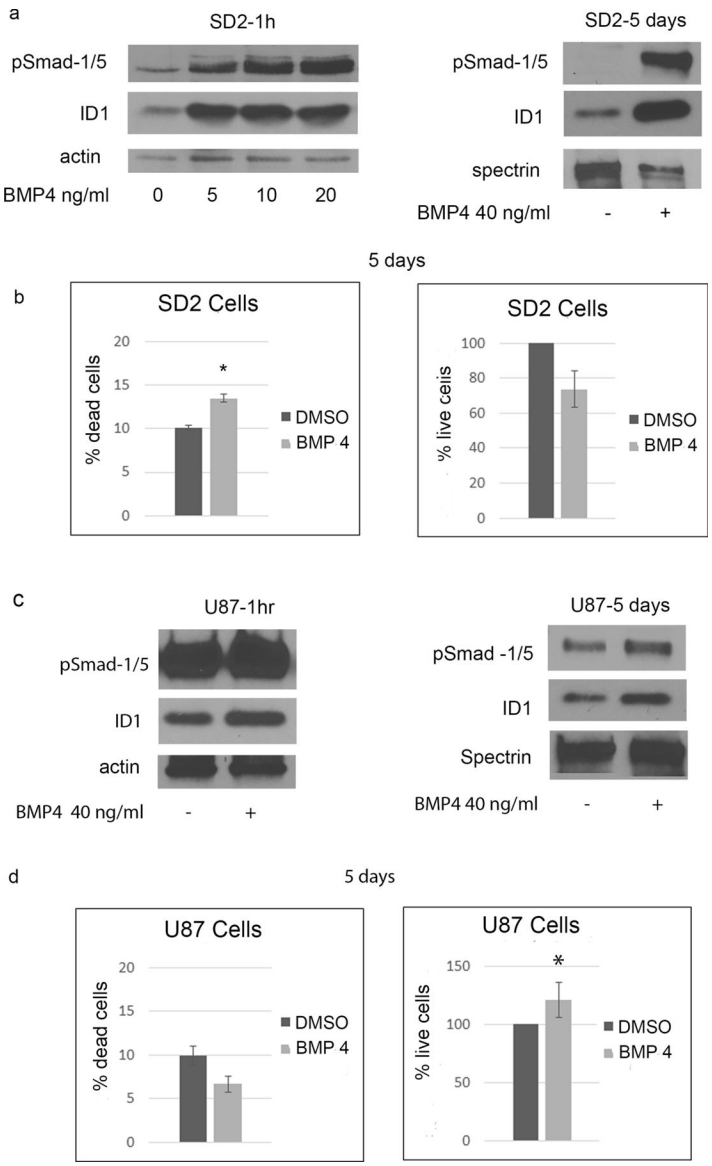

Figure 4. BMP4 activates ID1 expression and suppresses growth of SD2 cells.

(a) Western blot analysis of SD2 cells treated with BMP4. (b) SD2 cells were treated with BMP4 for 5 days and percentage of dead cells and number of live cells determined. Data represents the mean of 4 independent experiments. (c) Western blot analysis of U87 cells treated with BMP4. (d) U87 cells were treated with BMP4 for 5 days and percentage of dead cells and number of live cells determined. Data represents the mean of 4 independent experiments. * $p < 0.05$, compared to control.

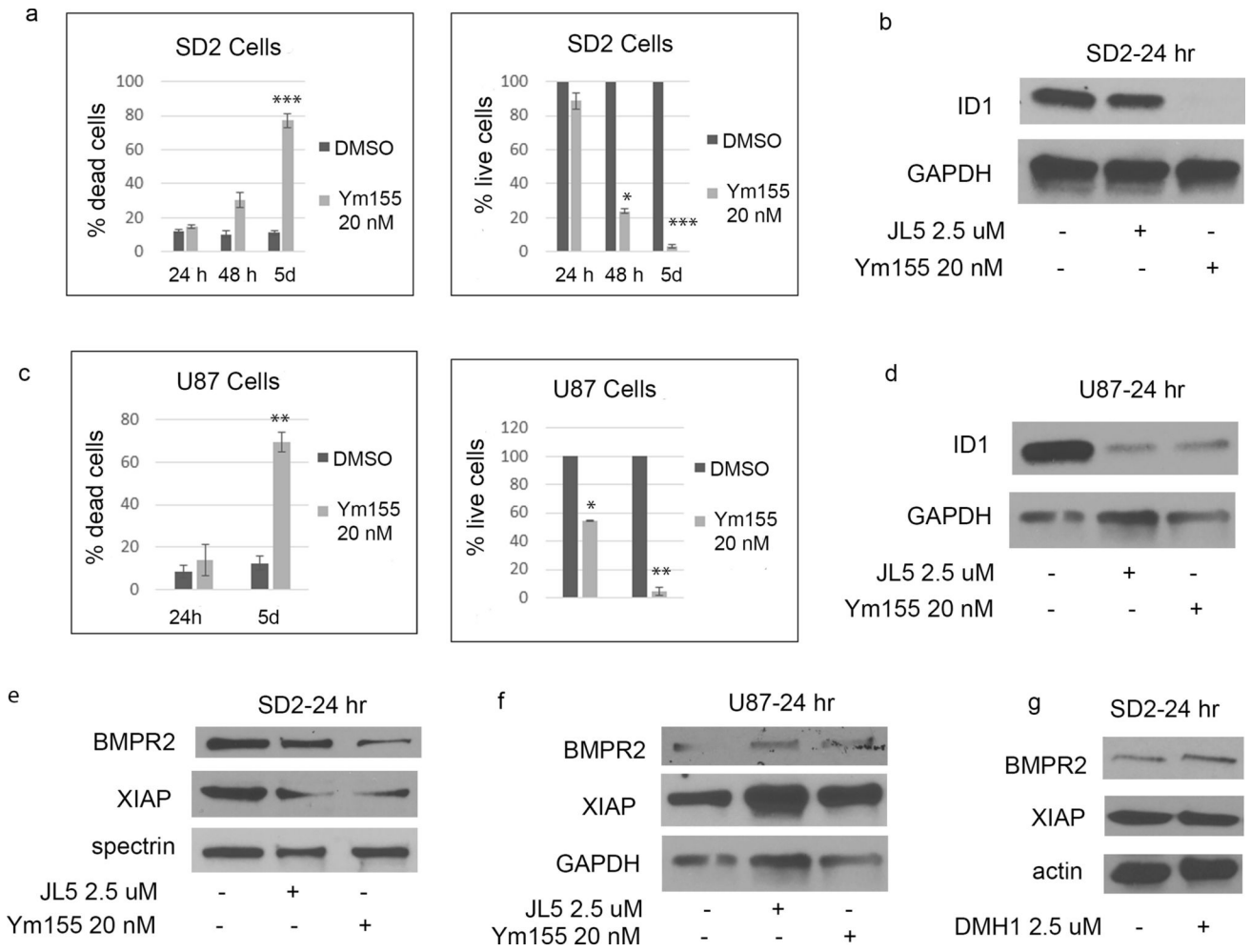

Figure 5. Ym155 inhibits BMP signaling. J5 and Ym155 decrease XIAP expression in SD2 cells.

(a,c) SD2 and U87 cells were treated with Ym155 and the percentage of dead cells and number of live cells determined. Data represents the mean of 4 independent experiments. (b,d) Western blot analysis of SD2 and U87 cells treated with Ym155 or JL5 demonstrating a decrease in ID1 expression. (e,f) Western blot analysis of cells treated with JL5 and Ym155 showing a decrease the expression of XIAP in SD2 but not the U87 cells. (g) Western blot analysis showing DMH1 does not decrease expression of XIAP. * $p < 0.05$, ** $p < 0.005$, *** $p < .0005$ compared to control.

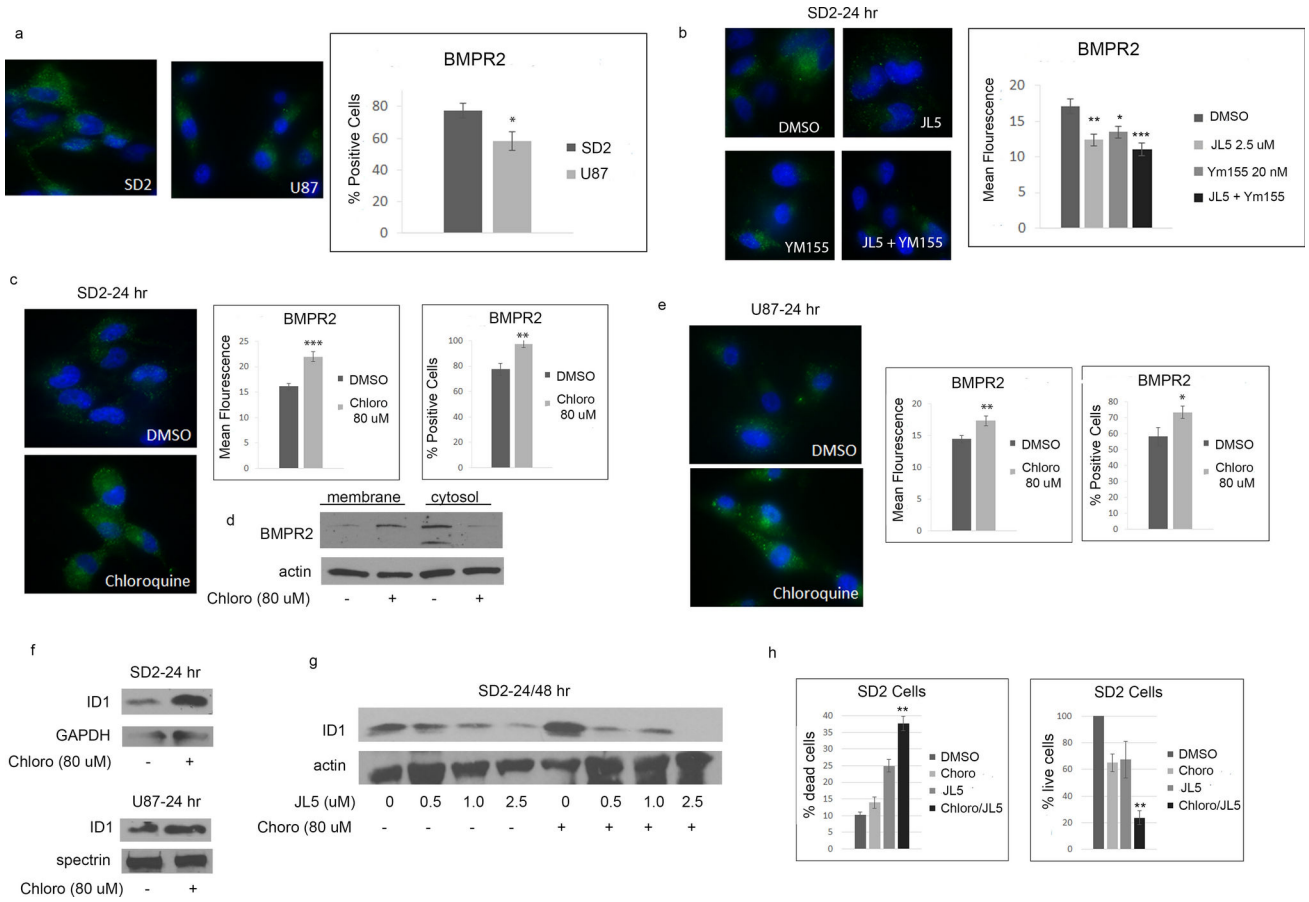

Figure 6. Chloroquine increases BMPR2 expression and enhances cell death induced by JL5.

(a) Representative immunofluorescent images of BMPR2 in untreated cells. Graphs represent the percentage of cells expressing BMPR2. Approximately 80 cells were counted from each cell line. (b) Representative immunofluorescent images of BMPR2 following treatment with JL5 and Ym155. Graphs represent the mean fluorescence of approximately 20 cells from each treatment group. (c,e) Representative immunofluorescent images of BMPR2 following treatment with chloroquine. Graphs represent the mean fluorescence of approximately 30 cells from each treatment group and the percentages of cells that express BMPR2. (d) Western blot of membrane and cytosol fraction of SD2 cells treated with chloroquine for 24 hours. (f) Western blot showing chloroquine increases the expression of ID1. (g) Western blot analysis showing that chloroquine enhances JL5 induced down-regulation of ID1. (h) Cell counts of SD2 cells pre-treated with either DMSO or Chloroquine for 24 hours, then treated again with either DMSO or JL5 for 48 hours. The data represents the mean of 4 independent experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ compared to control. (g) ** $p < 0.005$ chloroquine + JL5 in comparison to JL5 alone.

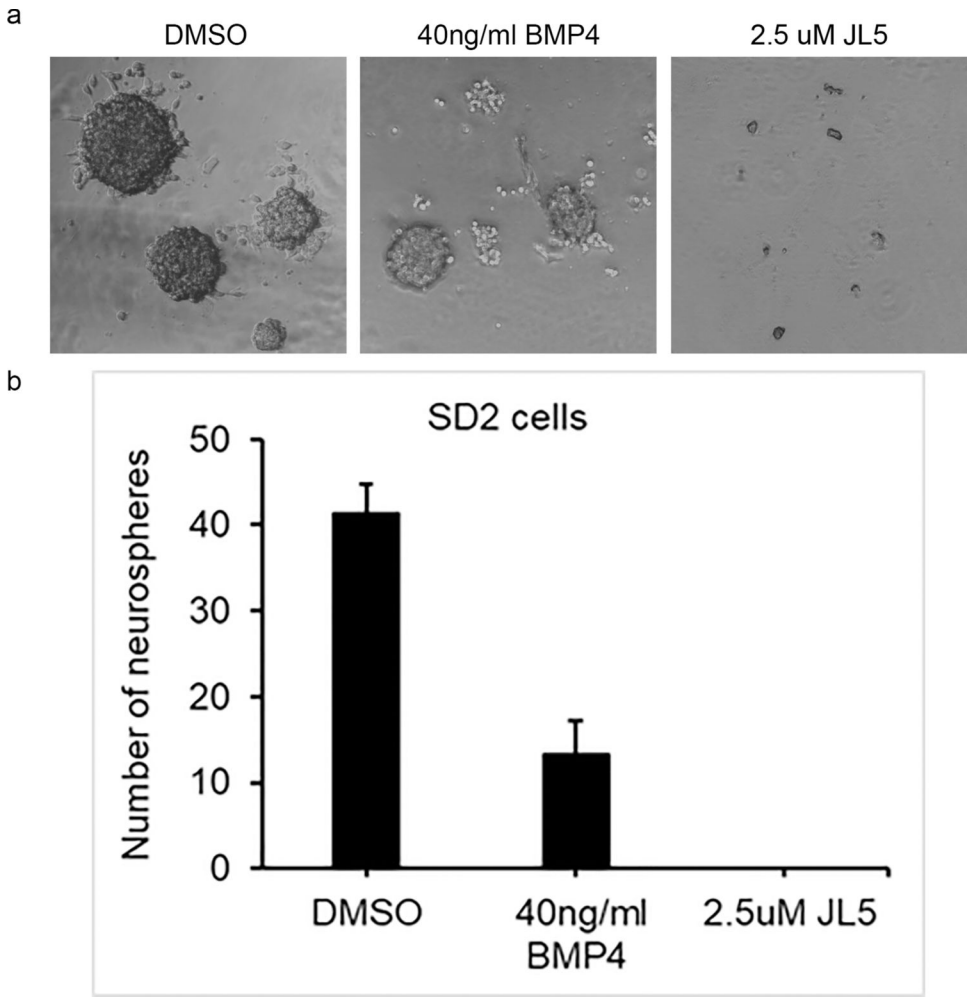

Figure 7. JL5 inhibits self-renew more than BMP4.

1000 SD2 cells were plated into 6 well plates and then treated with BMP4 or JL5 for 7 days. (a) Representative phase contrast images of sphere formation. (b) All the spheres in the 6 well plates were counted. Depicted is the mean number of spheres from 3 independent experiments.