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BMP Signaling Induces Astrocytic Differentiation of Clinically-derived Oligodendroglioma Propagating Cells

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Abstract

Oligodendrogliomas are a type of glioma that lack detailed investigation due to an inability to cultivate oligodendroglioma cells that faithfully recapitulate their salient qualities. We have successfully isolated and propagated glioma stem-like cells from multiple clinical oligodendroglioma specimens. These oligodendroglioma propagating cells (OligPCs) are multipotent and form xenografts with oligodendroglioma features. Bone morphogenetic proteins (BMPs) are considered potent inhibitors of oligodendroglial development; therefore, the effects of BMP signaling in OligPCs were characterized. BMP pathway components are expressed by OligPCs and canonical signaling via Smad proteins is intact. This signaling potentially depletes CD133-positive OligPCs, decreasing proliferation and inducing astrocytic differentiation. Furthermore, analyses revealed that cytoplasmic sequestration of the oligodendrocyte differentiation factors OLIG1/2 by the BMP signaling effectors ID2 and ID4 is a plausible underlying mechanism. These findings elucidate the molecular pathways that underlie the effects of BMP signaling on oligodendroglioma stem-like cells.

Keywords

Oligodendroglioma; bone morphogenetic proteins; glioma stem-like cells; differentiation

Introduction

Thorough characterization of the cells capable of propagating gliomas is critical for the development of more effective therapies. Glioblastomas, the most malignant of these tumors, are thought to be propagated by glioma stem cells (GSCs), a subpopulation of cells with neural stem cell (NSC) properties (1–3). The cells responsible for initiating oligodendrogliomas, however, remain controversial. Mouse models overexpressing the key signaling molecule PDGF-B in nestin-positive neural progenitors give rise to oligodendrogliomas (4–6), suggesting that NSCs are capable of serving as the cell-of-origin

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Conflicts of Interest:

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for these tumors. Indeed, stem-like cells have recently been isolated from two anaplastic human oligodendrogliomas (7). However, other populations of cells have also been successfully used to initiate oligodendrogliomas. For instance, co-expression of the oncogenes RAS and EGFRvIII in GFAP-positive astrocytes results in the formation of murine oligodendrogliomas (8), and overexpression of EGFR in white matter oligodendrocyte progenitor cells (OPCs) similarly induces hyperplasia resembling oligodendrogliomas (9). NG2-positive OPCs also yielded oligodendrogliomas when exposed to a transplacental mutagen (10). In keeping with these findings, OPCs, and not NSCs, have recently been proposed as the cell-of-origin for human oligodendroglioma as well (11). Interestingly, PDGF overexpression in CNPase-positive OPCs yields primarily grade II oligodendrogliomas (12), in contrast with the more malignant tumors produced by viral transduction of NSCs, suggesting that cell of origin may dictate tumor grade. A better understanding of the cells capable of propagating oligodendrogliomas will facilitate a more detailed exploration of their signaling, and subsequently the development of therapeutics targeting the important factors identified.

One signaling pathway of particular interest is the bone morphogenetic protein (BMP) pathway. BMPs are secreted factors that act through direct binding to a heterotetramer of membrane-bound BMP family receptor kinases, leading to phosphorylation and binding of cytosolic Smad proteins, translocation to the cell nucleus, and ultimately gene expression (13). BMPs exert pleiotropic effects, with specificity conferred by cell type and developmental context (14). Importantly, BMPs are potent inhibitors of oligodendroglial lineage specification in both neural stem cells and oligodendrocyte progenitor cells, directing them instead towards an astrocytic fate (15, 16). This aspect of BMP signaling remains intact in glioma stem cells (GSCs) isolated from glioblastomas, as BMP treatment results in reduced proliferation and increased expression of astrocyte-specific markers, suggesting the induction of differentiation (17, 18). Importantly, BMP treatment also decreases tumorigenicity of GSCs *in vivo* (17, 19), suggesting that BMPs may be exploited as a GSC-targeted therapy in these tumors. The molecular pathways responsible for these effects, however, are not fully understood.

Here, we describe the establishment of glioma stem-like cells from multiple different human oligodendrogliomas. We further demonstrate that BMP signaling is intact in these cells, and potently induces their astrocytic differentiation. Finally, we reveal cytoplasmic sequestration of oligodendrocyte lineage transcription factors (OLIG) 1 and 2 by BMP-induced ID proteins as a putative mechanism underlying this effect. Our findings have important implications for the development of therapies targeting the stem-like cell compartment of oligodendrogliomas.

Materials and Methods

Oligodendroglioma propagating cell isolation and culture

OligPCs were isolated from primary surgical specimens from patients with known or suspected oligodendroglioma in keeping with protocols approved by the Northwestern University Institutional Review Board and grown as spheres as previously described (2). In brief, specimens were rinsed in 1x phosphate-buffered saline (PBS), mechanically

dissociated with a scalpel and enzymatically dissociated using DNaseI (Roche) and Dispase (GIBCO) in DMEM/F12 media (Invitrogen) at 37°C for 45 min. Red blood cells were lysed using ACK buffer (Gibco), and a single cell suspension was achieved using a 100 µm strainer. Cells were plated in non-adherent flasks in DMEM/F12 containing 1% penicillin/streptomycin, supplements N2 and B27 (Gibco), and the following growth factors: 20 ng/ml human recombinant EGF (Millipore), 20 ng/ml bFGF (Millipore) and 10 ng/ml LIF (Chemicon). Once spheres were visible, cell cultures were centrifuged at 100 x g for 5 minutes and the supernatant was aspirated to remove dead cells and cellular debris as needed. Such centrifugation was often performed multiple times before the spheres were passaged.

The final diagnosis for each tumor, including lineage-specific immunohistochemical stains and fluorescent in situ hybridization confirming the characteristic 1p19q chromosomal deletion, was obtained before cells were used in subsequent experiments. OligPC 40 was derived from a primary WHO grade III oligodendroglioma with 1p19q chromosomal deletion and polysomy for chromosome 10. Areas of focal anaplasia with increased proliferative index were apparent, and no astrocytic features were observed. OligPC 49 was derived from a recurrent WHO grade III oligodendroglioma also with 1p19q chromosomal deletion. This tumor demonstrated frequent mitoses and microvascular proliferation, as well as marked cellular atypia, with some cells resembling typical oligodendroglial cells and other with enlarged nuclei or multiple nuclei. However, no astrocytic component was apparent upon immunohistochemistry for GFAP. Mutations in IDH1 and IDH2 were not assessed in these tumors, as the pathological analyses were performed prior to the identification of these mutations in oligodendroglial tumors (20).

OligPC spheres were passaged every 7–10 days by mechanical chopping. Cells were used at passage 10 or less for all experiments. For sphere-forming assays, cells were plated in 96-well plates at a density of 10 cells/well in 100µl GSC media. After 10 days, each well was inspected for sphere formation, and the number of spheres per well were counted.

Clonogenic frequency was estimated as the average number of spheres formed per 100 cells plated. For differentiation assays, cells were dissociated to a single cell suspension using Accutase (Sigma) and plated on glass coverslips coated with poly-D-lysine/laminin (BD Biosciences) and grown in GSC media without growth factor supplementation. For cultures with BMP treatment, human recombinant BMP4 (R&D Systems) was added to a final concentration of 100 ng/ml.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Sigma) in 1x PBS for 20 min, washed 3 times in PBS, and incubated with primary antibodies overnight at 4°C in 1x PBS containing 1% bovine serum albumin and 0.25% Triton X-100. After 3 more PBS washes, cells were incubated with the appropriate secondary antibody (Molecular Probes, Invitrogen) at 1:500 in 1x PBS for 1 h at room temperature. Nuclei were counterstained with Hoechst dye (1:5000 in 1x PBS), coverslips were mounted using Prolong Gold antifade reagent (Invitrogen) and imaged on a Zeiss UV-LSM 510 META or Leica SP-5 confocal microscope. National Institutes of Health Image J software was used to quantify images. The

following primary antibodies were used: MAP2 (Abcam, mouse IgG1, 1:1000), GFAP (DakoCytomaton, rabbit polyclonal, 1:1000), O4 (Millipore, mouse IgM, 1:100 in 1x PBS; incubated with cells at room temperature for 30 min prior to fixation), Sox2 (Millipore, rabbit polyclonal, 1:500), Nestin (BD biosciences, mouse IgG1, 1:500), Ki67 (Chemicon, mouse IgG1, 1:500), phospho-Smad1/5/8 (Cell Signaling, rabbit polyclonal, 1:500), ID2 (Santa Cruz, rabbit polyclonal, 1:100), ID4 (Santa Cruz, rabbit polyclonal, 1:50), OLIG1 (Millipore, mouse IgG2b, 1:250), OLIG2 (Millipore, mouse IgG2a, 1:250). Cell viability and death were determined using a LIVE/DEAD Viability/Cytotoxicity kit, which labels live cells using green-fluorescent calcein-AM and dead cells using red-fluorescent ethidium homodimer-1, per manufacturers instructions (Molecular Probes).

Protein isolation, Immunoprecipitation and Western blotting

OligPCs were lysed in M-PER protein extraction reagent (Pierce) supplemented with 1x HALT Protease + Phosphatase inhibitor cocktail (Thermo Scientific). For immunoprecipitation, a protein A immunoprecipitation kit was used per manufacturer's instructions (Roche). For Western blot analyses, samples were boiled for 10 min and loaded on 4–20% SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes at 4°C for 1 h, which were then blocked in TBS-T (Tris-buffered saline with 0.05% Tween-20) with 5% nonfat dry milk for 1 h at room temperature. Primary antibodies were diluted in blocking solution and incubated with membranes overnight at 4°C. After washing, membranes were incubated with the appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:2000 in blocking solution), washed three more times with TBS-T, and developed using SuperSignal West Pico ECL reagent (Thermo Scientific). The following primary antibodies were used: BMPR1A C-terminal (Abgent, rabbit polyclonal, 1:1000), BMPR1B (Abgent, rabbit polyclonal, 1:1000), BMPR2 (Abgent, rabbit polyclonal, 1:1000), ID2 (Santa Cruz, rabbit polyclonal, 1:500), ID4 (Santa Cruz, rabbit polyclonal, 1:500).

Flow cytometry

OligPCs were cultured with or without 100ng/ml BMP4 for 7 days. Cells were dissociated with Accutase, blocked in 10% FBS in 1x PBS, and incubated with either CD133/2–APC antibody or mouse IgG1-APC isotype control antibody (Miltenyi Biotec) in blocking solution on ice for 30 minutes. Cells were subjected to flow cytometric analysis of CD133 percentage with a CyAn machine (Beckman Coulter), with gating controls set using cells stained with the isotype control antibody.

RNA extraction and quantitative PCR (qPCR)

Total RNA from cultured cells was extracted using the RNAqueous-4PCR kit (Ambion), and 0.5µg of RNA was subjected to reverse transcription to produce cDNA using Thermoscript reverse transcriptase and oligo-dT primers (Invitrogen). qPCR was performed using 2µl (one tenth) of the cDNA reaction with SybrGreen master mix (Applied Biosystems) and a Realplex2 Mastercycler (Eppendorf) using the following cycling parameters: 95°C, 15 s; 60°C, 60 s for 40 cycles. The following primers were used: ID2: TGACCACCCTCAACACGGATATCAG, GCC ACACAGTGCTTTGCTGTC, ID4: CAAGCAGGGCGACAGCATTCT, TCTCTAGTGCTCCT GGCTCGG, BMPR1A:

TGGCTCGTCGTTGTATCACAGGAG, CCACCGATTAGACACAAT TGGCCG;
 BMPR1B: GGCCTCATCCTTTGGGAGGTT, GGTCAGTGGGCACTAGGTCA TG;
 BMPR2: CACAAATGTCCTGGATGGCAGCA, AGTGGAGATGACCCAGGTGGA;
 GAPDH: CTTCAACAGCGACACCCACTCC, GTCCACCACCCTGTTGCTGTAG.

Mouse intracranial xenografts

6–8 week old NOD-SCID-IL2R $\gamma^{-/-}$ (NSG) mice were obtained from The Jackson Laboratory and subsequently bred in the barrier animal facility at Northwestern University. All animal procedures adhered to Public Health Service Policy on Humane Care and Use of Laboratory Animals. Mice were injected with 1×10^5 OligPCs (passage 5) in 2 μ l of media in the right striatum as previously described (1), 6 mice per cell line. Mice were monitored daily for up to 6 months for the development of symptoms.

Tissue processing and immunohistochemistry

Tumor formation was assayed in frozen sections by either staining with hematoxylin and eosin or processing for immunohistochemistry in a manner similar to that described for the cells. 1X PBS containing 10% normal goat serum (10% FBS was substituted for samples stained with OLIG2 antibody) and 0.25% Triton X-100 was used as blocking buffer, and antigen retrieval was performed by incubating slides in 10mM sodium citrate at 95°C for 10 minutes. Primary antibodies used were: GFAP (DakoCytomaton, rabbit polyclonal, 1:1000), OLIG2 (R&D Systems, goat polyclonal, 1:1000), human-specific Nestin (BD Biosciences, mouse IgG1, 1:500).

Statistical Analyses

For immunofluorescence experiments, at least 200 cells per condition were counted. All experiments were performed independently 3 times and significance was assessed by two-tailed Student's *t* test.

Results

Isolation of human oligodendroglioma propagating cells (OligPCs)

The isolation and characterization of cancer stem cells from astrocytomas, medulloblastomas and ependymomas have been invaluable for elucidating their biology (1, 21), but there is only one brief report of culturing such cells from human oligodendroglioma (7). Therefore, we attempted to isolate stem-like cells from acutely dissociated primary oligodendroglial tumors. Spherical cellular aggregates formed in our oligodendroglioma cell cultures (Figure 1A), though they took much longer to form as compared to GBM cultures, usually 3–4 weeks. Substantially higher amounts of cellular debris were present in these cultures, and multiple media changes were required before spheres were readily detectable. These differences in sphere-forming latency and debris quantity may have led previous groups to prematurely conclude that their culture attempts were unsuccessful (11, 22). Of note, many of the cells that adhered to the flask and failed to form spheres demonstrated small dark nuclei and abundant cytoplasm similar to immature oligodendrocytic cells early in culture (Figure 1A, inset). We were able to successfully expand cultures of these oligodendroglioma propagating cells (OligPCs) using serial passaging for more than 6

passages, suggesting long-term self-renewal capacity well beyond the sphere-forming capacity generally ascribed to more differentiated transit amplifying cells (23) (Figure 1B). To more formally assess self-renewal capacity, clonal density single well sphere-forming assays were performed. OligPCs were able to form secondary spheres even at a density of 1 cell/10 μ l, with a clonogenic frequency of $6.4\pm0.58\%$, in keeping with previously reported glioma clonogenic frequencies (24). It should be noted that such spheres may also be formed by an aggregation of multiple cells, as has been observed in sphere-forming assays at higher densities (25), and thus may also represent non-clonal cell populations. Of the 10 oligodendroglial tumor samples from which we attempted to isolate stem-like cells, all but 2 formed primary spheres (80%). We were successfully able to propagate spheres more than six passages in 4 of these 8 lines (50%).

OligPCs demonstrate stem-like properties

As expression of NSC markers is traditionally used in identifying glioma stem cells, we evaluated the presence of various NSC markers in our OligPC lines by immunofluorescence. Nestin and sox2 were both strongly expressed by OligPCs (Figure 1C). We also examined expression of CD133 in our OligPC lines by flow cytometry, and observed a range of percentages of CD133-positive cells (Figure 1D). Thus, OligPCs express markers traditionally used to describe glioma stem cells.

Another defining characteristic of NSCs and GSCs is their ability to differentiate into cells of all three neural lineages. We investigated the multipotency of OligPCs by plating them on poly-D-lysine/laminin coverslips in media without exogenous mitogens for 7 days, followed by immunofluorescence for markers of different neural lineages. The OligPC lines were capable of generating neurons, astrocytes and oligodendrocytes under these conditions (Figure 2B), whereas they did not express these markers prior to differentiation (Figure 2A). Notably, such differentiation was detectable in cells originating from a single sphere derived at clonal density. Our OligPCs are thus multipotent, with the caveat that we did not test differentiation capacity at the single cell level, in contrast to previous reports suggesting more restricted lineage potential (11). We observed considerable co-expression of these lineage markers, particularly O4 and MAP2, which has also been observed in GSCs (26). However, many cells expressed only markers of oligodendroglia in the absence of other lineage markers (Figure 2C). In fact, OligPC lines readily generated cells in the oligodendroglial lineage, as evidenced by expression of multiple different markers of this cell fate (Figure 2D), which are only rarely observed in differentiated GSC cultures. These cells demonstrated marked elaboration of processes in a lacy pattern, reminiscent of mature oligodendrocytes. This suggests that oligodendrocyte differentiation programs may remain intact in these cells.

OligPCs form xenografts recapitulating oligodendroglioma features

A rigorous standard by which cancer stem cells are judged is the ability to form tumors that phenocopy the tumor of origin in immunocompromised mice. We thus injected two of our OligPC lines orthotopically into immunocompromised mice to determine their tumorigenic potential. Both OligPC lines that we tested *in vivo* were capable of tumor formation, though tumor latencies were longer than typically observed with GSCs (Figure 3A). This is

consistent with the slower proliferation rate of OligPCs observed in cell culture as well as the slower growth of oligodendrogliomas in the clinical context. These tumors demonstrated marked hypercellularity and cytoplasmic clearing with relatively well-demarcated margins (Figure 3B). OligPC-derived tumors also exhibited a propensity for spontaneous hemorrhage, which has been described in oligodendrogliomas (27). This may be due to the delicate “chicken-wire” vasculature that is a hallmark of the disease histopathology (Figure 3B).

Immunostaining with a human-specific nestin antibody confirmed the xenograft origin of the tumors, again demonstrating dense hypercellularity (Figure 3C). Of note, the intensity of nestin staining was somewhat less than that observed with GSC xenografts, which is consistent with the differential expression of nestin observed by others at the cellular level (11). We further characterized the xenograft tumors by staining with lineage-specific markers. OligPC-derived xenografts demonstrated strong nuclear expression of the oligodendroglial transcription factor OLIG2 (Figure 3D). By contrast, cells expressing the astrocytic protein GFAP were relatively scarce (Figure 3D). This distribution of oligodendroglial and astrocytic markers is diagnostic of oligodendrogliomas. Thus, as these OligPC lines form tumors characteristic of human oligodendrogliomas, they fulfill the criteria defining cancer stem-like cells.

BMP signaling is intact in OligPCs

Bone morphogenetic protein (BMP) signaling is a notable negative regulator of oligodendroglial development (28). Importantly, this pathway has also been implicated as an inhibitor of tumorigenicity in GSCs (17), where it induces astrocytic differentiation. We thus sought to elucidate the effect of BMP signaling in OligPCs. We first evaluated the expression of BMP receptor subunits in these cells using quantitative PCR. Transcripts for both type I subunits (BMPRI1A and BMPRI1B), as well as the type II subunit with which they dimerize (BMPRII), were detectable in OligPCs (Figure 4A). We confirmed BMP receptor expression at the protein level by Western blot analysis (Figure 4B). We next investigated the integrity of canonical BMP signaling in OligPCs. Cells were treated with BMP4 for 2 hours followed by immunofluorescence for phospho-Smad-1/5/8 (pSmad), the downstream mediator of canonical BMP signaling. While untreated OligPCs demonstrated considerable cytoplasmic localization of pSmad, OligPCs treated with BMP4 showed robust nuclear pSmad with almost no visible cytoplasmic protein (Figure 4C-C’). Quantification of this effect revealed a 2-fold increase in the number of cells exhibiting nuclear pSmad localization (Figure 4C, far right). Finally, we assayed induction of downstream target genes of BMP signaling, the inhibitor of differentiation/DNA binding (ID) family of proteins (29). Cells were grown in the presence or absence of BMP4 for 8 hours followed by isolation of RNA and evaluation of ID transcript expression by quantitative PCR. OligPCs treated with BMP4 demonstrated a 2 to 4-fold induction of ID2 and ID4 transcripts (Figure 4D) as compared to untreated cells. Thus, the canonical BMP signaling axis is functional in OligPCs.

BMP decreases proliferation and induces astrocytic differentiation of OligPCs

We next explored the biological effects of BMP signaling in OligPCs. We first assessed the effect of BMP treatment on OligPC growth kinetics. OligPCs were plated in medium containing normal mitogens (EGF, FGF and LIF) in the presence or absence of BMP4, and viable cell counts were obtained by trypan blue dye exclusion at 4 and 7 days after plating. Addition of BMP4 completely abrogated the expansion of OligPCs, yielding static viable cell numbers at both time points (Figure 5A, left). Interestingly, similar cell numbers were observed when OligPCs were cultured in the absence of mitogens (GSC media –E/F/L), indicating that BMP4 is able to completely counter the effects of mitogens on OligPC expansion. This effect did not seem to be due to cytotoxicity of BMP treatment, as there was no difference in the percentage of dead cells among the conditions at day 7 (Figure 5A, right). As BMPs are known to elicit mitotic arrest via their induction of the cyclin-dependent kinase inhibitor p21 (30), we examined the cell cycle status of OligPCs treated with BMP. OligPCs were grown in the presence or absence of BMP4 for 7 days, followed by immunostaining for Ki67. BMP reduced the percentage of mitotically active OligPCs by 30–50% (Figure 5B). Thus, similar to its effect on GSCs (17), BMP reduces OligPC number by slowing cell cycle transit.

As BMP treatment also results in a specific reduction in the CD133-positive subpopulation in GSCs, we evaluated the expression of this marker in OligPCs. As described above, OligPCs were cultured with or without BMP4 for 7 days, at which point they were subjected to flow cytometric analysis of CD133. BMP treatment resulted in a marked 5-fold decrease in the percentage of CD133-positive OligPCs (Figure 5C). Thus, BMP signaling is able to deplete the proportion of OligPCs expressing a putative cancer stem cell marker even in the presence of mitogens. This reduction in stemness was also observed functionally, using the clonal sphere-forming assay. BMP treatment reduced the clonogenic frequency of OligPCs by almost 50% (Figure 5D), confirming its potent inhibition of stem-like properties of OligPCs.

The diminished proliferation caused by BMP in NSCs is accompanied by the promotion of astrocytic lineage specification (15), and this phenotype is also observed upon BMP treatment of GSCs (17). To examine the potential for altered fate commitment in OligPCs as a result of BMP signaling, OligPCs were plated on poly-D-lysine/laminin coverslips in media without mitogens in the presence or absence of BMP4. After 7 days, cells were subjected to immunofluorescence for oligodendroglial and astrocytic markers. Untreated OligPCs demonstrated extensive elaboration of O4-positive processes, indicative of oligodendroglial differentiation. By contrast, relatively few GFAP-positive astrocytes could be visualized (Figure 6A). This finding is in keeping with our observation that OligPCs are more biased toward an oligodendroglial fate than their GSC counterparts (data not shown). Remarkably, BMP treatment resulted in a striking decrease in O4-positive oligodendrocytes and a concomitant increase in GFAP-positive astrocytes (Figure 6B,C). This effect was confirmed using alternate markers of oligodendroglial and astrocytic lineage specification, CNPase and S100 β , respectively (Figure 6D). These findings indicate that BMP signaling is sufficient to promote robust astrocytic differentiation of OligPCs, in contrast to previous reports suggesting no effect of BMP treatment on OligPC fate specification (11).

BMP-induced ID proteins interact with OLIG1/OLIG2

We have previously shown that the anti-oligodendroglial effects of BMP signaling on NSCs are mediated by induction of the ID family helix-loop-helix transcription factors ID2 and ID4, which subsequently sequester the oligodendrocyte transcription factors OLIG1 and OLIG2 in the cytoplasm, thereby preventing the transcription of genes promoting the oligodendroglial lineage (31). Given the potent induction of *ID2* and *ID4* by BMP in OligPCs (Figure 4D), we explored the possibility that a similar mechanism mediates the effect of BMP on lineage specification in OligPCs. Cells were cultured with or without BMP4 for 12 hours and then immunostained for ID and OLIG proteins. In the absence of BMP, OLIG1 is predominantly localized in the nucleus (Figure 7A,B). However, following BMP treatment, OLIG1 is predominantly localized in the cytoplasm, demonstrating profound co-localization with both ID2 (Figure 7A) and ID4 (Figure 7B). Similarly, OLIG2 localization shifts from nuclear to cytoplasmic with BMP treatment (Figure 7C, D). These results suggest that BMP4 may inhibit oligodendroglial specification of OligPCs via prevention of OLIG nuclear translocation by ID proteins, in keeping with the mechanism elucidated in oligodendrocyte progenitors. To more directly investigate an interaction between OLIG and ID proteins, we performed co-immunoprecipitation experiments in OligPCs treated with BMP4 for 12 hours. OligPC lysates were immunoprecipitated with OLIG1 and OLIG2 antibodies, followed by Western blot analyses with ID2 and ID4 antibodies. Both ID2 and ID4 were detectable after immunoprecipitation with either OLIG1 or OLIG2 (Figure 7E), indicating that ID2 and ID4 interact with the OLIG proteins in BMP-treated OligPCs. Thus, BMP signaling is able to potently inhibit oligodendroglial specification and promote astroglial specification in OligPCs, possibly via posttranslational inhibition of OLIG1/2 by ID2/4.

Discussion

Understanding the unique biology of oligodendrogliomas has been frustrated by a paucity of *in vitro* systems capable of recapitulating the hallmarks of this disease. Very few such oligodendroglia propagating cell lines have been reported, and their signaling pathways remain poorly characterized (7, 11). Here we report the isolation and molecular characterization of additional OligPC lines. Previous studies of stem-like cells isolated from oligodendrogliomas have yielded conflicting results with respect to the most basic characterization of these cells. Kelly et al reported sphere-forming capacity and multipotent differentiation (7), similar to our findings, suggesting that OligPCs exhibit properties of neural stem cells and GSCs isolated from astrocytomas. Other groups, however, have observed deficiencies in the ability of OligPCs to form spheres (22), and reported a more restricted capacity for differentiation (11). These findings, combined with their observation that OligPC tumorigenicity is enriched in NG2-positive cells but not CD133-positive cells, led Persson et al to conclude that OligPCs are more closely related to oligodendrocyte precursor cells rather than neural stem cells. Interestingly, the tumors used by Persson et al were both grade II oligodendrogliomas, whereas our cells were derived from more aggressive oligodendrogliomas, as were the cells used by Kelly et al. CD133 expression is strongly correlated with tumor grade in astrocytomas (32), and a CD133-associated gene signature has been used to identify a more aggressive subtype of glioblastoma (33),

suggesting that multipotent self-renewing tumor cells may be more prevalent in higher grade gliomas. Thus, high-grade oligodendrogliomas may be propagated by stem-like cells resembling NSCs, as characterized in this study, while low-grade oligodendrogliomas are propagated by the more restricted precursor cells described by Persson et al. Indeed, varying cells-of-origin paralleling stages in neurogenesis have been demonstrated in astrocytomas (34), providing proof-of-concept for analogous disease progression in oligodendrogliomas. More careful dissection of the relationship between glioma initiating cells and eventual glioma grade will help clarify this issue.

BMP signaling promotes astrocytic differentiation of normal NSCs (15), and this differentiation effect is conserved in GSCs isolated from glioblastomas, resulting in decreased tumorigenicity (17, 18). The present study, to our knowledge, presents the first comprehensive exploration of BMP signaling in human OligPCs. We show that BMP treatment is sufficient to reduce proliferation and stemness in OligPCs, instead promoting astrocytic fate commitment. As these findings mirror the observations made in astrocytoma GSCs, it is likely that BMP treatment would result in decreased tumorigenicity of OligPCs as well. Our findings stand in contrast to previous reports, where no effect of BMP treatment on OligPC differentiation was observed (11), although those cells differ from the cells used in this study, as detailed above. In addition, differential responses to BMP treatment have been observed in glioblastoma GSCs as a result of epigenetic silencing of the *BMPR1B* promoter (35). As no examination of BMP receptor expression was carried out by Persson et al, it is difficult to assess this possibility. However, it is conceivable that a similar mechanism exists in OligPCs, and that methylation of the *BMPR1B* promoter in the OligPCs studied by their group accounts for the disparate response they observed. Our study suggests OligPC differentiation via BMP treatment as a viable novel approach to targeted therapy in anaplastic oligodendrogliomas, perhaps taking advantage of newly-developed small molecule BMP agonists (36). An assessment of the tumorigenicity of BMP-treated OligPCs in immunocompromised mice, which was not within the scope of this study, would be of paramount interest.

While a role for BMP signaling in the inhibition of gliomagenesis has been established, the mechanisms underlying this effect have not yet been elucidated. In this study, we delineate one such potential mechanism, involving the antagonism of OLIG1 and OLIG2 proteins by the BMP-induced helix-loop-helix proteins ID2 and ID4 (Figure 7). Indeed, ID proteins are known to bind to other such lineage specific basic-helix-loop-helix transcription factors to inhibit their activity (37). OLIG2 has been proposed as a key mediator of gliomagenesis (38–40), and downregulation of OLIG2 is required for inhibition of glioblastoma tumorigenicity by BMP7 (41). ID2 and ID4 have been implicated in this process, as they promote astroglial differentiation in GSCs by downregulating OLIG1/2 (42). The pro-migratory transcription factor Snail is also induced by BMP7 in GSCs, and Snail overexpression decreases GSC tumorigenicity but increases xenograft invasiveness (43), highlighting the complexity of the molecular pathways activated by BMPs. Importantly, OLIG2 is also indispensable for tumor propagation in a PDGF-B-driven mouse model of oligodendroglioma, and the diminished tumorigenic capacity caused by loss of OLIG2 is mimicked by ID4 upregulation (44). These results, in combination with our findings, suggest

BMP-mediated inhibition of OLIG1/2 as a promising avenue of targeted therapy for oligodendroglioma.

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Implications

Stem-like cells are capable of propagating oligodendrogliomas, and BMP signaling potently diminishes their stemness by inducing astrocytic differentiation, suggesting that BMP activation may be effective as a cancer stem cell-targeted therapy.

Figure 1. Human oligodendrogloma propagating cells (OligPCs) form spheres and express NSC markers

(A) Brightfield image of primary spheres formed by dissociated oligodendrogloma cells in GSC conditions. Adherent cells with oligodendrocytic morphology are evident (inset, arrow). Scale bar: 200 μm .

(B) Brightfield image of OligPC spheres formed after serial passaging, demonstrating self-renewal. Scale bar: 500 μm .

(C) OligPCs immunostained for NSC markers sox2 and nestin, with DAPI nuclear counterstain; merged image at far right. Scale bar: 50 μm .

(D) Flow cytometry for CD133 in two different OligPC lines.

Figure 2. OligPCs are multipotent and readily generate oligodendrocytes

- (A) Undifferentiated OligPCs were allowed to attach to PDL coverslips and stained for the oligodendrocyte marker O4, the neuronal marker MAP2, and the astrocytic marker GFAP, with DAPI nuclear counterstain. Scale bar: 50µm.
- (B) OligPCs were differentiated on PDL/laminin coverslips in media without growth factors and stained for O4, MAP2, and GFAP, merged with DAPI nuclear counterstain at bottom right. A representative confocal z-stack is shown. Scale bar: 200µm.
- (C) OligPCs differentiated as above and stained for MBP and MAP2. Scale bar: 50µm.
- (D) OligPCs differentiated as above and stained for multiple oligodendrocyte lineage markers: O4 (left), myelin basic protein (MBP, center), and CNPase (right). Scale bars: 50µm.

Figure 3. OligPCs form tumors that resemble oligodendrogliomas in vivo

(A) Kaplan-Meier survival curves showing tumorigenicity of 2 OligPC lines following orthotopic transplantation in 6 mice each.

(B) H&E staining of an OligPC xenograft shows cytoplasmic clearing, spontaneous hemorrhage (arrow), and fine branching vasculature (arrowhead). Dashed line: tumor margin. Magnification: 10 $\times$.

(C) Immunostaining with a human-specific nestin antibody confirms xenograft origin and demonstrates invading cells (arrowheads) beyond the tumor margin (dashed line). Scale bar: 100 μ m.

(D) Immunostaining with lineage markers reveals robust OLIG2 expression with a relative paucity of GFAP expression in xenografts; merged with DAPI nuclear counterstain at far right. Scale bar: 100 μ m.

Figure 4. BMP signaling remains intact in OligPCs

- (A) Quantitative RT-PCR for BMP receptor subunits in 2 OligPC lines, relative to GAPDH control. Shown as mean+SEM.
- (B) Western blot for BMP receptor subunits in 2 OligPC lines, with GAPDH loading control, quantified at right.
- (C) OligPCs were incubated with or without 100ng/ml BMP4 for 2 hours and immunostained for phospho-Smad-1/5/8, with DAPI nuclear counterstain. Scale bars: 50µm. Zoomed images of the boxed areas demonstrate cytoplasmic (C') and nuclear (C'') pSmad localization. Scale bars: 20µm. Quantification of this staining, shown as mean+SEM, is at far right, **p<0.005 by two-tailed Student's t-test.

(D) Quantitative RT-PCR for ID2 (left) and ID4 (right) transcripts in OligPCs treated with 100ng/ml BMP4 for 8 hours, normalized to GAPDH control and shown as mean+SEM relative to untreated cells. * $p < 0.05$, ** $p < 0.005$ by two-tailed Student's t-test.

Figure 5. BMP signaling reduces proliferation and stemness of OligPCs

(A) OligPCs were grown in GSC media, media without mitogens, or media+BMP4 and total viable cells were counted using Trypan blue, shown as mean $\pm$ SEM (left). ** $p < 0.05$ by two-tailed Student's t-test. OligPCs grown in these conditions were also subjected to a live/dead assay, and the percentage of dead cells at day 7 was calculated, shown as mean $\pm$ SEM (right).

(B) OligPCs were grown with or without 100ng/ml BMP4 for 7 days and cell cycle status was assessed by Ki67 staining with DAPI nuclear counterstain, shown as mean $\pm$ SEM at far right. Scale bar: 50 μ m. * $p < 0.01$ by two-tailed Student's t-test.

(C) OligPC 40 was grown as in (B) and CD133 expression was assessed by flow cytometry, shown as mean $\pm$ SEM at far right. * $p < 0.01$ by two-tailed Student's t-test.

(D) OligPC 49 was plated at 10 cells/well in 96 well plates with or without 100ng/ml BMP4 for 10 days, and the clonogenic frequency was calculated by counting the number of spheres formed, shown as mean+SEM. ** $p < 0.05$ by two-tailed Student's t-test.

Figure 6. BMP signaling induces astrocytic differentiation of OligPCs

(A) OligPCs were grown with or without 100ng/ml BMP4 for 7 days, followed by immunofluorescence for markers of oligodendroglial (O4) and astrocytic (GFAP) fate, with DAPI nuclear counterstain. Scale bar: 50μm.

(B+C) Quantification of the staining shown in (A) for OligPC 49 (B) and OligPC 40 (C), shown as mean+SEM, *p<0.01 and **p<0.02 by two-tailed Student's t-test.

(D) OligPCs were grown as in (A), followed by immunofluorescence for alternate markers of oligodendroglial (CNPase) and astrocytic (S100β) fate, with DAPI nuclear counterstain (scale bar: 50μm), with quantification shown as mean+SEM at far right, *p<0.01 and **p<0.02 by two-tailed Student's t-test.

Figure 7. OLIG proteins co-localize with ID2 and ID4 in BMP-treated OligPCs

(A–D) OligPCs were grown with or without 100ng/ml BMP4 for 12 hours, followed by immunostaining for OLIG1 + ID2 (A), OLIG1 + ID4 (B), OLIG2 + ID2 (C) and OLIG2 + ID4 (D), with DAPI nuclear counterstain. Scale bars: 50µm. Zoomed images of the boxed areas demonstrate nuclear (A') and cytoplasmic (A'') Olig localization. Scale bars: 25µm.

(E) OLIG1 or OLIG2 protein was immunoprecipitated from OligPCs grown as in (A–D) and Western blot analysis was performed for ID2 and ID4.