

Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation

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Summary

Bone morphogenetic protein (BMP) signaling inhibits the generation of oligodendroglia and enhances generation of astrocytes by neural progenitor cells both in vitro and in vivo. This study examined the mechanisms underlying the effects of BMP signaling on glial lineage commitment. Treatment of cultured neural progenitor cells with BMP4 induced expression of all four members of the inhibitor of differentiation (ID) family of helix-loop-helix transcriptional inhibitors and blocked oligodendrocyte (OL) lineage commitment. Overexpression of *Id4* or *Id2* but not *Id1* or *Id3* in cultured progenitor cells reproduced both the inhibitory effects of BMP4 treatment on OL lineage commitment and the stimulatory effects on astroglialogenesis. Conversely, decreasing the levels of *Id4* mRNA by RNA interference enhanced OL differentiation and inhibited the effects of BMP4 on glial lineage commitment. This suggests that induction of *Id4* expression mediates effects of BMP signaling. Bacterial two-hybrid

and co-immunoprecipitation studies demonstrated that ID4, and to a lesser extent ID2, complexed with the basic-helix-loop-helix transcription (bHLH) factors OLIG1 and OLIG2, which are required for the generation of OLs. By contrast, ID1 and ID3 did not complex with the OLIG proteins. In addition, the OLIG and ID proteins both interacted with the E2A proteins E12 and E47. Further, exposure of cultured progenitor cells to BMP4 changed the intracellular localization of OLIG1 and OLIG2 from a predominantly nuclear to a predominantly cytoplasmic localization. These observations suggest that the induction of ID4 and ID2, and their sequestration of both OLIG proteins and E2A proteins mediate the inhibitory effects of BMP signaling on OL lineage commitment and contribute to the generation of astrocytes.

Key words: Neural stem cell, BMP4, Oligodendroglia, ID, Olig, Astrocyte, Mouse

Introduction

Although neural progenitor cells competent to differentiate into oligodendrocytes (OLs) are widely distributed in the early embryonic central nervous system (CNS), the earliest OL progenitors arise in relatively discrete ventral regions along the neural axis (Orentas and Miller, 1996; Qi et al., 2002; Wada et al., 2000). Over time, OLs become more widespread either by migration and/or by a process of progressive inductive signaling. In the forebrain, the progeny of bipotent glial progenitors within the subventricular zone (SVZ) adopt an astrocytic or oligodendroglial fate depending on their post-migratory environment. Sonic Hedgehog (Shh) released from the notochord and floor plate is primarily responsible for the initial specification and production of OLPs in the ventral spinal cord (Orentas et al., 1999). Bone morphogenetic proteins (BMPs) are expressed in the CNS during the time of gliogenesis, particularly in dorsal regions, and in vivo inactivation of BMP signaling results in dorsal expansion of OL precursors (OLP) (Hardy and Friedrich, 1996; Mekki-Dauriac et al., 2002). This suggests that the anatomic restriction of oligodendroglial development is regulated by a balance between the positive ventral effects of Shh signaling and the dorsal inhibitory effects of BMP signaling.

BMP signaling exerts different effects on lineage

commitment by neural stem/progenitor cells at different stages of development. Early in mouse cortical development (E13) BMP signaling promotes neuronal lineage commitment by cultured neural progenitor cells (Li et al., 1998; Mabie et al., 1999), whereas later in development (E16 and thereafter) it promotes astrocytic differentiation of ganglion eminence progenitor cells (Gross et al., 1996; Mehler et al., 1995; Zhu et al., 1999a), cortical progenitor cells (Mabie et al., 1999) and O2A progenitor cells (Grinspan et al., 2000; Mabie et al., 1997). However, at all stages of development BMP signaling consistently inhibits oligodendroglial lineage commitment by cultured progenitor cells (Gross et al., 1996; Mehler et al., 1995; Zhu et al., 1999a). Conversely, treatment of cultured glial progenitors with noggin, an inhibitor of BMP signaling, promotes oligodendrogenesis (Mabie et al., 1999). Furthermore, transgenic overexpression of BMP4 enhances astrocyte lineage commitment in vivo and significantly inhibits the generation of OLs (Gomes et al., 2003), whereas overexpression of the BMP inhibitor, noggin, under the same promoter conversely increases the number of oligodendroglia and inhibits astroglialogenesis (J.A.K., unpublished).

The molecular mechanisms by which BMPs inhibit oligodendrogenesis are not known. BMP signaling upregulates expression of the inhibitor of differentiation (Id) family of

proteins (Miyazono and Miyazawa, 2002) in diverse cell types such as neuroepithelial cells (Nakashima et al., 2001), osteoblasts (Ogata et al., 1993) and embryonic stem cells (Hollnagel et al., 1999). The ID family of proteins includes four related helix-loop-helix transcription factors (*Id1-Id4*) which do not contain the basic DNA binding regions adjacent to the helix-loop-helix dimerization domain (Norton et al., 1998). Basic helix-loop-helix (bHLH) factors can be broadly classified into two groups, Class A factors and Class B factors. The class A factors, or E proteins (E2-2, HEB, and the E2A gene products E12 and E47), are expressed ubiquitously and are able to dimerize with tissue-specific class B bHLH factors to activate gene expression (Massari and Murre, 2000). ID proteins sequester the class A factors, inhibiting the formation of active class A-class B heterodimers and thus act as dominant negative regulators of differentiation (Benezra et al., 1990; Sun et al., 1991). However, the IDs may also bind to some class B bHLH factors involved in muscle differentiation such as MyoD and Myf-5 (Langlands et al., 1997).

Cells of the OL lineage express IDs and the bHLH factors OLIG1 and OLIG2 (Lu et al., 2000; Zhou et al., 2000). In *Olig1/2* double-mutant mice, there is a complete failure of OL development in all areas of the brain along with an apparent increase in astrocytogenesis in the spinal cord (Zhou and Anderson, 2002). This indicates that *Olig1/2* expression is essential for oligodendroglial development and suggests that repression of OL development may be sufficient to cause astrocytogenesis. Expression of *Id4* in OL precursor cells progressively decreases as the precursor cells differentiate in vivo and in vitro (Kondo and Raff, 2000). Overexpression of *Id2* inhibits OL differentiation and its absence induces premature OL differentiation in vitro (Wang et al., 2001).

This study shows that ID2 and ID4 directly interact with OLIG1 and OLIG2 while all the ID proteins act to sequester the E2A proteins. Overexpression of *Id4* or *Id2* or treatment with BMP4 inhibits OL lineage commitment even in cells that overexpress *Olig1* and *Olig2*, and enhances astrocytogenesis. Conversely, inhibition of *Id4* expression by RNA interference (RNAi) blocks the inhibitory effects of BMP4 on OL lineage commitment. These observations suggest that ID4 and ID2 sequestration of OLIG proteins mediates the inhibitory effects of BMP signaling on OL lineage commitment and underlies the restriction of oligodendroglial development to ventral regions of the nervous system where Shh signaling predominates (Orentas and Miller, 1996; Qi et al., 2002; Wada et al., 2000).

Materials and methods

Cell culture and transfections

Dissociated E17 lateral ganglionic eminence (CD1 mice, Taconic) cells (5×10^4 per milliliter) were grown in DMEM/F12 with N2 and B27 nutrient additives and EGF (10 ng/ml) (BD Biosciences) for 7 days to generate neurospheres. To optimize expression of oligodendroglial precursors, neurospheres were dissociated and cultured in EGF (10 ng/ml) (BD Biosciences), NT3 (10 ng/ml) (Sigma) and PDGF-AA (10 ng/ml) (Sigma) for 3 days prior to experimental use. For phenotypic analysis, cells were grown on Poly-D-lysine (PDL)-coated coverslips for 2 days in 2 ng/ml EGF with or without 20 ng/ml BMP4 (R&D). For overexpression experiments, cells were infected with lentivirus containing the respective genes by following the manufacturer's protocol in lentiviral expression kit (Invitrogen). We modified the D-Lenti-TOPO vector by inserting an

IRES-GFP sequence. Viral titers obtained were 5×10^5 on an average, and approximately 70% cells were infected.

Immunocytochemistry

Cells cultured on coverslips were fixed with 4% paraformaldehyde. Mouse monoclonal antibodies against 2'3' cyclic nucleotide phosphodiesterase (CNPase) 1:800 (Sternberger Monoclonals), myelin basic protein (MBP) 1:600 (Sternberger Monoclonals), glial fibrillary acid protein (GFAP) 1:400 (Sigma), β -tubulinIII 1:400 (Sigma), anti-chk GFP antibody 1:3000 (Chemicon), NG2 1:400 (Chemicon) and OLIG2 1:500 (UT Southwestern) or rabbit polyclonal antibodies against OLIG1 1:500 (Chemicon), ID2 and ID4 1:50 (Santa Cruz) were applied overnight at 4°C. Then the appropriate fluorophore conjugated-secondary antibodies 1:1000 (Molecular Probes) were applied and the nuclei were counterstained with DAPI. Controls were performed without primary antibody, with alternate primary antibodies, and with inappropriate secondary antibodies to show negligible background. Total cellular counts for each experimental condition were obtained by examining the entire area of each coverslip from three independent culture wells; the result for each experimental condition was verified a minimum of three times. For double staining OLIG1 and IDs, zenon rabbit antibody labeling kit (Molecular Probes) was used.

Bacterial two-hybrid assay

The manufacturer's protocol in the bacterial two-hybrid kit (Stratagene) was followed.

Co-immunoprecipitation (Co-IP)

Two hundred and ninety-three cells were co-transfected with pCDNA-ID1-4 and pCDNA-OLIG1/2, using Fugene6 transfection reagent (Roche) and Co-IP was performed according to manufacturer's recommendations in the protein G immunoprecipitation kit (Roche). Antibodies used for CO-IP were specific anti-IDs (1:50) (Santa Cruz) and anti-his6 antibody (1:150) (Invitrogen) for OLIGs. Western analyses of the precipitates were performed for the other interacting protein using the following antibodies: specific anti-IDs (1:200) (Santa Cruz), anti-OLIG1 (1:200) (Chemicon), anti-OLIG2 (1:200) (UT Southwestern) and anti-myc 9E10 supernatant (1:10) (DSHB). For detection of endogenous protein-protein interactions, E17 EGF-generated neurosphere cells treated with BMP4 (20 ng/ml) for 12 hours were lysed followed by CO-IP with anti-OLIG1, specific anti-IDs and normal rabbit IgG (1:50) (Santa Cruz).

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

QRT-PCR was performed using Perkin-Elmer's ABI Prism 7700 Sequence Detector System. Total RNA was extracted from cells using Trizol reagent (Invitrogen). cDNA was prepared using the thermoscript RT-PCR kit (Invitrogen). QRT-PCR was performed with an initial denaturation of 10 minutes at 95°C, followed by 40 cycles of 15 seconds' denaturation at 95°C and 1 minute of annealing and elongation at 60°C. SYBR green 1 dye was used to produce the fluorescent signal that was detected at the annealing phase. Specificity of the PCR reaction was confirmed by running PCR products on 2% agarose gel. Two replicates were run for each cDNA sample with the test and control primers in separate wells of a 96-well plate. An amplification plot showing cycle number versus the change in fluorescent intensity was generated by Sequence Detector program.

RNA interference (RNAi)

Oligonucleotides were designed such that a loop sequence TTCAAGAGA was placed in-between sense and antisense sequences corresponding to AAGN₁₈TT in the coding sequence of ID4. The sense and antisense oligonucleotides with 5' phosphates and PAGE purification were annealed and cloned into pLentilox 3.7 (gift from

Luk Van Parijs). Lentivirus was made by transfecting HEK293T cells with pLentilox 3.7 plasmid and packaging plasmids. After the virus was concentrated and titered, 5×10^5 viral particles were used to infect approximately 10^5 neural stem cells. Cells were grown as neurospheres for 48 hours after viral infection and then dissociated cells were plated on PDL-coated coverslips. Viral titers were 5×10^6 on average and approximately 80% progenitor cells were infected. Phenotypic analysis was done after 48 hours.

Results

BMP4 inhibits specification of oligodendrocytes from neural progenitor cells

To define effects of BMP4 on oligodendroglial lineage specification, we optimized expression of oligodendroglial precursors by culturing neurospheres in the presence of neurotrophin3 (NT3) and platelet derived growth factor AA (PDGF-AA) for 3 days before switching to defined media and plating the cells with or without BMP4. Culture of cells in the presence of PDGF and NT3 amplifies the number of oligodendroglial progenitor cells by inducing their proliferation and preventing differentiation, which is subsequently promoted after switching the medium (Barres and Raff, 1994; Calver et al., 1998; Mehler et al., 1995). We examined the phenotype of the cells in these cultures by immunostaining (Fig. 1) for neurons (β -tubulinIII), astrocytes (GFAP), OLPs (OLIG1 and NG2), immature OLs (CNPase) and mature OLs (MBP). Shortly after plating the cells (Fig.

1B) (8 hours), the cultures contained a large population of OLIG1⁺ cells (50.27% of cells) and a smaller population of NG2⁺ cells (10%), indicating the presence of oligodendroglial progenitor species. There was also a small population of CNPase⁺ cells (9.7%) that were also OLIG1⁺, indicative of a more differentiated population of OL lineage species. There were few MBP⁺ cells but there were populations of GFAP⁺ (3.78%) and β -tubulinIII⁺ cells (6.1%). Culture for 56 hours in the absence of BMP4 (Fig. 1B) resulted in OL maturation with a significant increase in the number of MBP⁺ cells (10.44%) without a reduction in the number of CNPase⁺ cells (10.58%) but with a significant reduction in the number of OLIG1⁺ cells (33.33%). At this point the majority of OLIG1⁺ cells were also immunoreactive for CNPase and/or MBP. There were no significant changes over this time period in numbers of GFAP⁺ or β -tubulinIII⁺ cells. By contrast, as previously reported for SVZ progenitor cells (Gross et al., 1996; Mehler et al., 1995; Zhu et al., 1999a), oligodendrocyte progenitor cells (Grinspan et al., 2000; Mabie et al., 1997) and cortical progenitors (Mabie et al., 1999; Marmur et al., 1998; Nakashima et al., 2001; Zhu et al., 1999b), we found a striking reduction in the number of oligodendroglial lineage cells in the presence of BMP4 coupled with a large increase in the number of GFAP⁺ cells (23.71%). There was no detectable difference in cell survival (live/dead staining) or overall cell numbers between the control and the BMP4-treated groups. The percentage of GFAP⁺ cells continued to increase with time in the presence of BMP4 without any increase in the number of OLPs or OLs (not

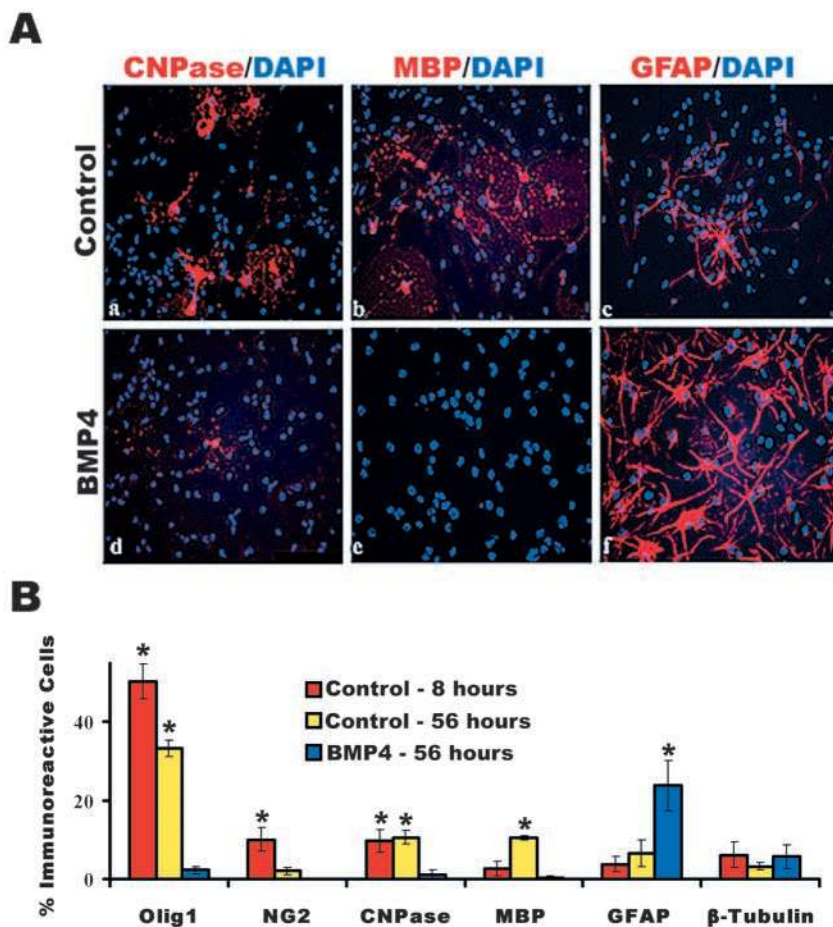


Fig. 1. BMP4 inhibits oligodendrocyte (OL) lineage commitment by neural progenitor cells. (A) EGF-generated neurosphere cells from E17 mice (A,B) were grown for 3 days in 10 ng/ml each of NT3, PDGF-AA and EGF. Cells were then dissociated and plated in media containing 2 ng/ml EGF in absence (A, parts a-c) or presence of 20 ng/ml BMP4 (A, parts d-f). Cells were stained for CNPase (A, parts a,d), MBP (A, parts b,e) and GFAP (A, parts c,f). (B) Cells were immunostained with OLIG1, NG2, CNPase, MBP, GFAP, and β -tubulinIII, and the mean numbers of immunoreactive cells were determined. BMP4 treatment significantly reduced the number of all OL species while increasing the number of astrocytes. Scale bars represent means \pm s.e.m. of four independent experiments. Anova was done to determine significance (* $P < 0.05$).

shown) (see Gross et al., 1996; Mehler et al., 1995; Zhu et al., 1999a; Nakashima et al., 2001).

In addition to the bHLH factors *Olig1* and *Olig2*, oligodendroglial progenitors express several transcription factors including the homeodomain protein *Nkx2.2* and the HMG domain protein *Sox10*. We performed QRT-PCR to examine effects of BMP4 on these transcription factors after 2, 8, 12, 24, 30 and 35 hours of exposure (Fig. 2A). Treatment of cultured E17 progenitor cells with BMP4 resulted in no change in transcript levels at 8 hours, suggesting a lack of a direct effect on gene expression. However, thereafter there was a gradual reduction of RNA levels of all the transcription factors, with a 60-fold decrease after 35 hours (Fig. 2A). This is consistent with the shift in phenotype of the cultured cells away from OL lineages.

BMP4 induces the expression of ID family of genes

To help define mechanisms underlying the effects of BMP signaling on lineage commitment, we next sought to identify

the genes regulated by BMP4 in cultured neural progenitor cells. A microarray analysis was performed to compare the levels of 9000 genes in cultured E17 progenitor cells treated with BMP4 for 2 hours with those in untreated control cells. Only seven genes, one of which was *Id4*, were upregulated more than 5-fold by 2 hours of exposure to BMP4. We confirmed our microarray finding by performing quantitative RT-PCR (Fig. 2B) to examine levels of mRNAs for the *Id* genes in E17 progenitor cells at 2, 8, 24 and 35 hours after BMP4 exposure. *Id* gene expression increased with BMP4 treatment as early as 8 hours after exposure. However, levels of *Id4* mRNA increased more than 16-fold, whereas levels of *Id1-3* mRNAs showed more modest increases (4-6 fold) after 8 hours of BMP4 treatment as compared with control levels. Levels of all ID proteins were also significantly elevated by treatment with BMP4 (Fig. 2C). These results suggest that BMP4 treatment increases expression of ID proteins concomitant with the suppression of oligodendrogenesis.

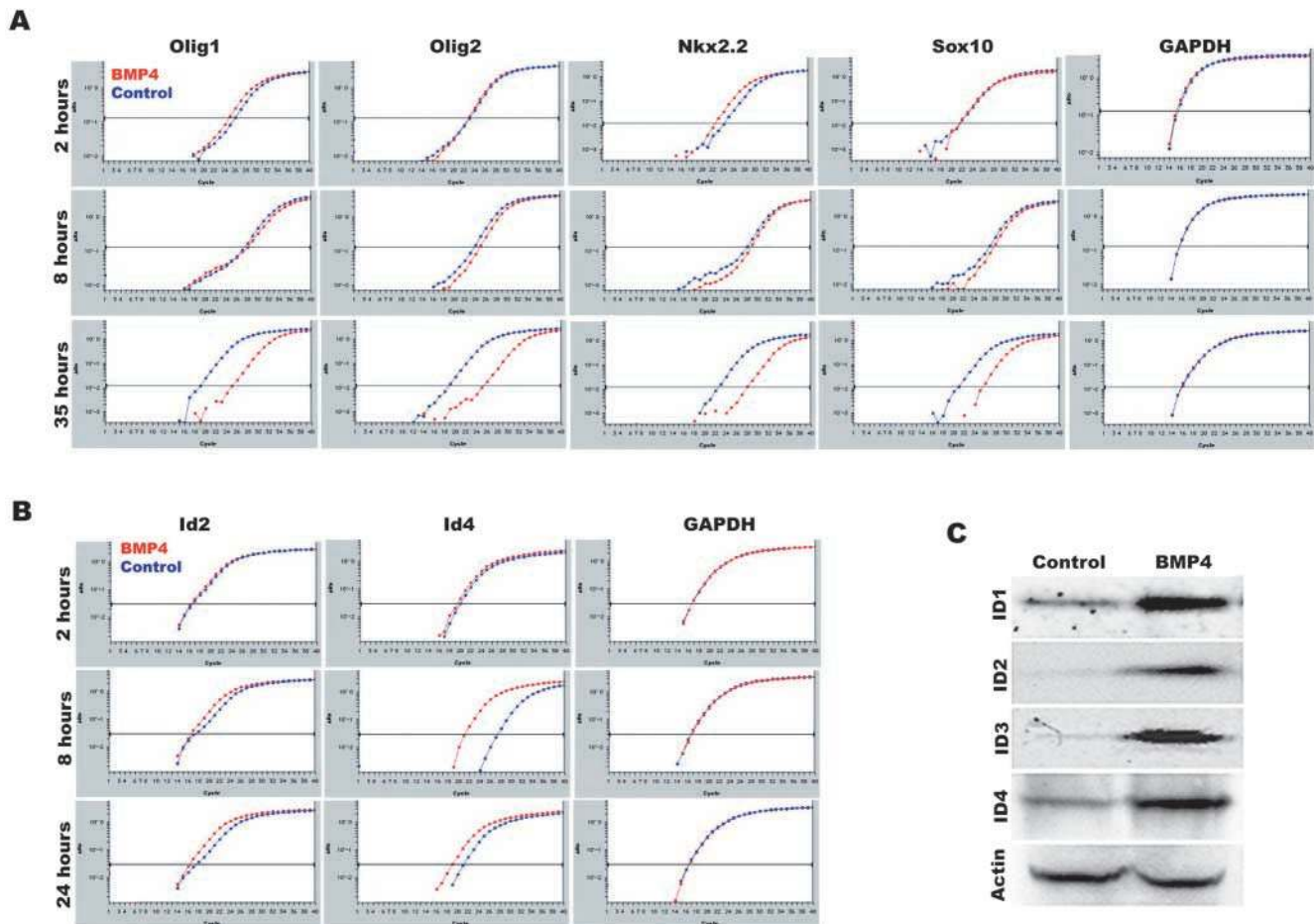


Fig. 2. BMP4 decreases expression of OL transcription factors and increases expression of ID genes. (A) Expression of mRNAs for *Olig1*, *Olig2*, *Nkx2.2* and *Sox10* was analyzed by quantitative RT-PCR performed with mRNA extracted from E17 progenitor cells grown in absence (blue) or presence (red) of 20 ng/ml BMP4 for 2, 8 and 35 hours. BMP4 treatment did not reduce levels of the transcripts until 8 hours. However, by 35 hours all transcripts were reduced by as much as 60-fold. (B) Expression of *Id2* and *Id4* mRNAs was analyzed by quantitative RT-PCR performed with mRNA extracted from E17 progenitor cells grown in absence (blue) or presence (red) of 20 ng/ml BMP4 for 2, 8 and 24 hours. *Id4* and *Id2* transcripts were both induced by BMP4 at 8 hours and subsequent time points. Graphs shown are representative of experiments done in duplicate three times. (C) Western blot analyses were performed to detect ID1-4 proteins using cell lysates from E17 progenitor cells grown in the absence (control) or presence of BMP4 for 12 hours. BMP4 treatment increased the expression of all the ID proteins.

ID2 and ID4 regulate the fate of neural progenitor cells

To determine whether the IDs might mediate effects of BMP4 on lineage commitment, we compared effects of lentiviral overexpression of *Id2* and *Id4* with the effects of treatment with BMP4 in cultured E17 progenitor cells. Levels of overexpressed IDs were confirmed by western blot analysis (Fig. 3A). Levels of ID4 appeared to be higher than ID2, although this could simply reflect differences in the antibodies used for blotting. Forty-eight hours after infection with the lentivirus, cultures were analyzed by immunofluorescence for neuronal (β -tubulinIII), astrocytic (GFAP) and oligodendrocytic (CNPase and MBP) markers (Fig. 3B). As noted previously, treatment with BMP4 reduced the numbers of CNPase⁺ and MBP⁺ cells by more than 95% and increased the number of GFAP⁺ cells by 2.5-fold (Fig. 3C). Overexpression of *Id2* reduced the number of CNPase⁺ cells by only approximately 25% and the number of MBP⁺ cells by approximately 40% and increased the number of GFAP⁺ cells 2.5-fold. However, overexpression of *Id4* resulted in a

significant 80% reduction in the number of CNPase⁺ cells and almost a 90% reduction in the number of MBP⁺ cells while increasing GFAP⁺ cells approximately 2.5-fold. Combined overexpression of *Id2* and *Id4* did not differ significantly from overexpression of *Id4* alone, and none of the treatments significantly altered the number of β -tubulinIII⁺ cells. Overexpression of *Id1* or *Id3* did not alter oligodendroglial or astrocytic lineage commitment (data not shown). These results suggest that *Id4* and *Id2* could act as downstream effectors of BMP4 in producing the inhibition of oligodendrogenesis and enhancement of astrocytic lineage commitment.

We tested this hypothesis further by inhibiting *Id4* expression using RNAi. Introduction of an *Id4*-RNAi oligonucleotide into E17 neural progenitor cells using lentivirus inhibited the expression of *Id4* mRNA in cells cultured with BMP4 (Fig. 4A). The effectiveness of RNAi in lowering levels of *Id4* mRNA was further confirmed by quantitative RT-PCR done on E17 progenitor cells treated with BMP4 for 12 hours (Fig. 4B), which showed approximately 95% reduction in levels of *Id4* mRNA. Infection of E17

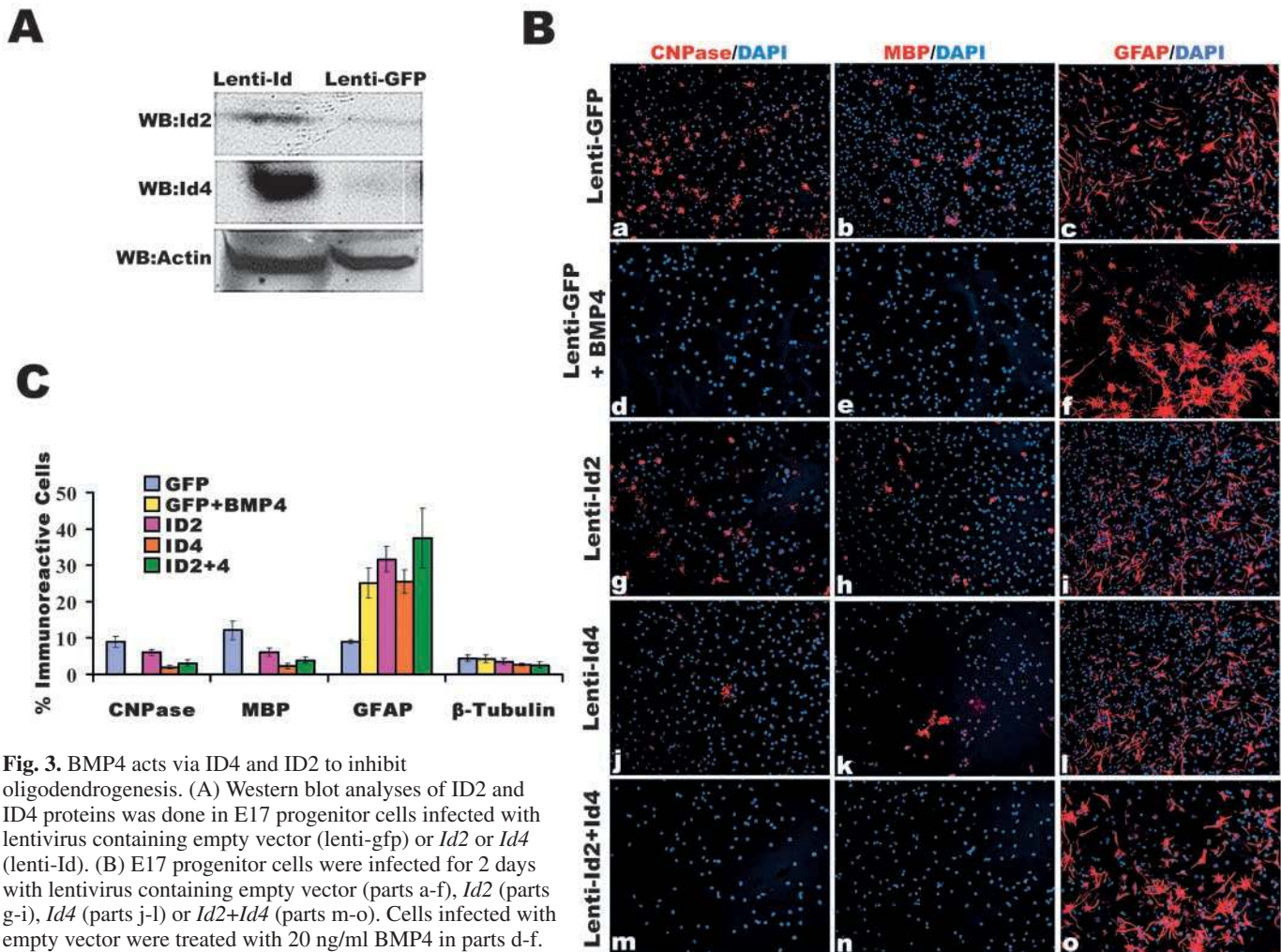


Fig. 3. BMP4 acts via ID4 and ID2 to inhibit oligodendrogenesis. (A) Western blot analyses of ID2 and ID4 proteins was done in E17 progenitor cells infected with lentivirus containing empty vector (lenti-gfp) or *Id2* or *Id4* (lenti-Id). (B) E17 progenitor cells were infected for 2 days with lentivirus containing empty vector (parts a-f), *Id2* (parts g-i), *Id4* (parts j-l) or *Id2+Id4* (parts m-o). Cells infected with empty vector were treated with 20 ng/ml BMP4 in parts d-f. Immunofluorescence was done for CNPase (parts a,d,g,j,m), MBP (parts b,e,h,k,n) and GFAP (parts c,f,i,l,o). DAPI was used to stain the nuclei. (C) Quantification for CNPase, MBP, GFAP and β -tubulinIII shows that ID overexpression has effects similar to those of BMP4 treatment, i.e. reduced numbers of OLs and increased numbers of astrocytes. Scale bars represent means \pm s.e.m. of four independent experiments. ANOVA was done to determine significance ($P < 0.05$). The control group (vector alone) differed significantly from other groups in numbers of CNPase, MBP and GFAP immunoreactive cells. There were no significant differences among β -tubulinIII groups.

progenitor cells with *Id4*-RNAi in the absence of BMP4 slightly increased the number of oligodendroglia and reduced the number of astrocytes, suggesting inhibition of baseline levels of *Id4*. Moreover, infection with *Id4*-RNAi blocked the BMP4-mediated suppression of oligodendroglial lineage commitment as well as the BMP4-mediated increase in astrocytes (Fig. 4C,D). These findings indicate that effects of BMP4 on lineage commitment are mediated by ID proteins, specifically ID4 and to a lesser extent ID2.

OLIG proteins interact with ID and E2A proteins

We next sought to define mechanisms by which ID4 and ID2

inhibit oligodendroglial lineage commitment. ID proteins lack a basic DNA binding domain and act as dominant negative inhibitors of bHLH transcription factors to which they bind. One way ID2 and ID4 could produce their effects is by directly interacting with OLIG1 and OLIG2 which are class B bHLH transcription factors that are required for oligodendroglial lineage commitment (Lu et al., 2000; Zhou et al., 2000). To examine interactions between ID and OLIG proteins, we performed co-immunoprecipitations with overexpressed ID and myc-his6-tagged OLIG proteins in HEK293T cells (Fig. 5A). OLIG1 and OLIG2 complexed with both ID2 and ID4 but larger bands were observed for ID4 (Fig. 5B). Importantly, we observed similar interactions between endogenous levels of OLIG and ID2 or ID4 proteins in E17 progenitor cells (Fig. 6B,C). The OLIG proteins did not interact with ID1 and ID3 in our assays (data not shown) but we did observe interactions between ID1 or ID3 and the known binding protein E12. To examine whether OLIG proteins interact directly with ID2 and ID4 without the need for other mammalian proteins, we performed a bacterial two-hybrid assay with the *Olig* genes in bait vectors and the *Id* genes in target vectors (Table 1). There were negligible numbers of colonies in the negative control plates but a large number of colonies in the test plates, indicating positive interactions of both OLIG proteins with ID2 and ID4. No such interactions were detected for ID1 or ID3 (not shown). This suggests that OLIG1 and OLIG2 directly interact with both ID2 and ID4 (Table 1) and that post-translational modification of either protein is not necessary for binding to occur. We next examined whether *Oligs* and *Ids* were co-expressed in progenitor cells (Fig. 6A). Both OLIG1 and OLIG2 colocalized with ID2 and ID4 in the cultured cells. In the absence of BMP4, OLIG2 was localized predominantly in the nucleus irrespective of the presence or absence of PDGF. However, after BMP4 treatment OLIG2 was localized predominantly in the cytoplasm in association with ID4 and ID2. Similarly,

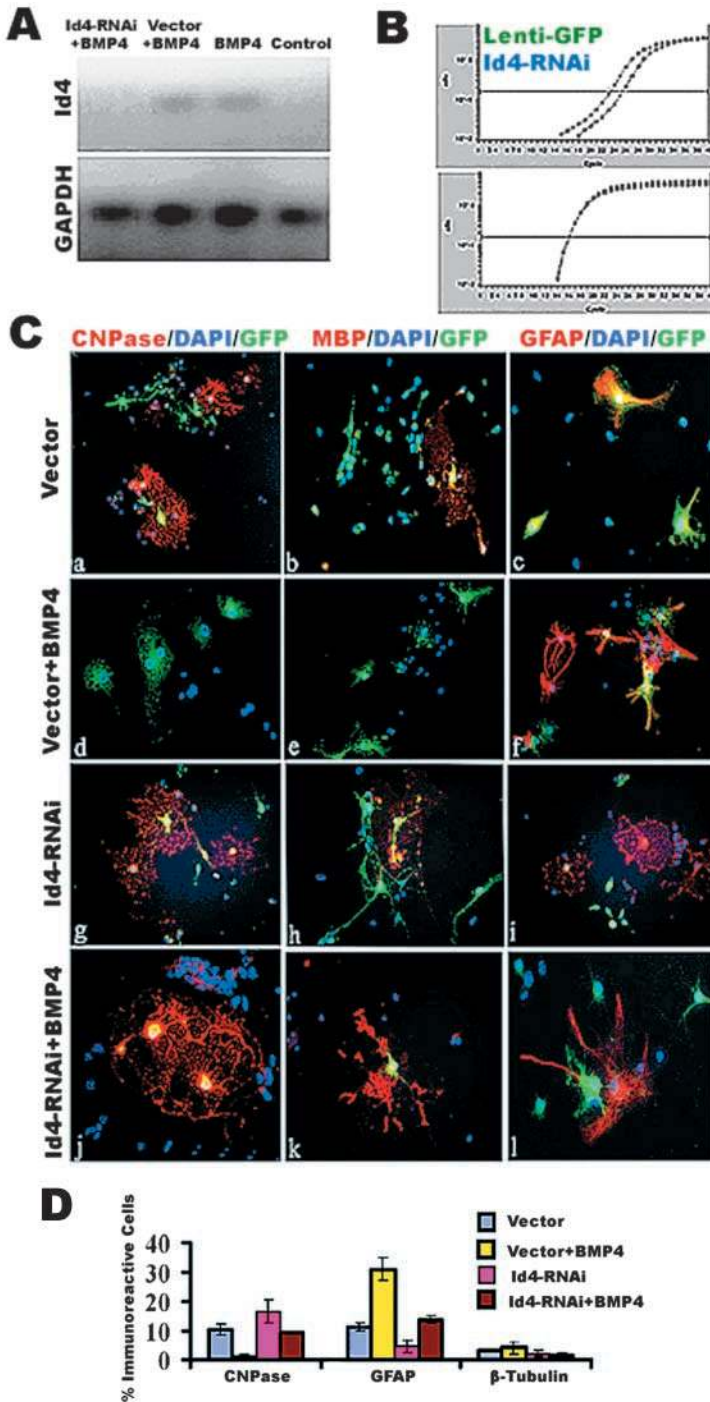


Fig. 4. Inhibition of ID4 prevents the effects of BMP4 on glial lineage commitment. The effectiveness of inhibition of *Id4* expression by RNAi was confirmed by RT-PCR (A) and quantitative RT-PCR (B) using mRNA extracted from E17 progenitor cells treated with BMP4 (20 ng/ml) for 12 hours and infected with lentivirus containing empty vector (green in B) or *Id4*-RNAi constructs (blue in B). The graphs shown are representative of experiments done in duplicate three times. (C) E17 progenitor cells infected with lentivirus containing empty vector (parts a-f) or *Id4*-RNAi (parts g-l) were grown with (parts d-f,j-l) or without (parts a-c,g-i) BMP4 (20 ng/ml) for 2 days. Cells were stained for CNPase, MBP and GFAP. DAPI was used as a nuclear stain and virus-infected cells were identified by staining for GFP. (D) Quantification of CNPase⁺, GFAP⁺ and β -tubulinIII⁺ cells shows increased numbers of OLs with *Id4*-RNAi. Scale bars represent means \pm s.e.m. of four independent experiments. ANOVA was done to determine significance ($P < 0.05$). The control (vector alone) group differed significantly from both BMP4-treated and *Id4*-RNAi groups with respect to CNPase and GFAP, but did not differ from the group treated with both BMP4 and *Id4*-RNAi. The BMP4-treated and *Id4*-RNAi-treated groups each differed from all other groups with respect to CNPase and GFAP. There were no significant differences among groups with respect to β -tubulinIII.

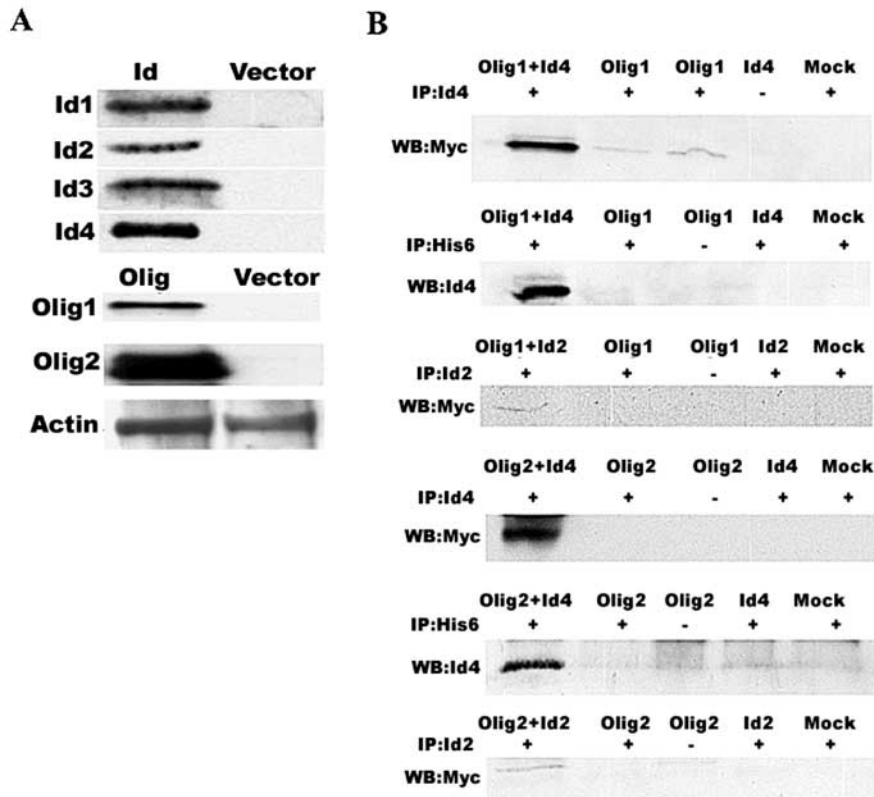


Fig. 5. OLIG proteins interact with ID2 and ID4 in HEK293T cells. (A) ID1-4 and OLIG1-2 proteins were overexpressed in HEK293T cells. (B) Co-immunoprecipitations were done with protein lysates from HEK293T cells transfected with *Olig1/2*-myc-his6 and *Id1-4*. OLIG proteins were pulled down with anti-his6 antibody and immunoblotted with anti-myc antibody, whereas specific ID antibodies were used to pull down or immunoblot IDs.

after BMP4 treatment OLIG1 was localized predominantly in the cytoplasm. This suggests that the interaction between the ID and OLIG proteins occurs in the cytoplasm and may prevent translocation of the OLIG proteins to the nucleus.

Oligodendroglial progenitors express E2A proteins, e.g. E12 and E47, which belong to the class A family of bHLH transcription factors (Sussman et al., 2002). ID proteins are typically thought to function as transcriptional repressors by sequestering class A factors and thus inhibiting dimerization of the ubiquitously expressed class A factors with tissue-specific class B bHLH transcription factors (Benezra et al., 1990; Langlands et al., 1997; Loveys et al., 1996; Sun et al., 1991). Class A and class B heterodimers recognize a consensus sequence in the enhancer elements of several bHLH factor-regulated genes. Hence, a second mechanism of inhibiting oligodendrogenesis could be the sequestration of E2A proteins by IDs. Although the IDs are known to interact with E2A proteins, such an interaction has not been shown for the OLIG proteins. We performed co-immunoprecipitations with overexpressed E2A and myc-his6-tagged OLIG proteins in HEK293T cells and found that both OLIG1 and OLIG2 interact with the E2A proteins, E12 and E47 (Fig. 7A,B). In all, these results suggest that the IDs inhibit oligodendrogenesis by two mechanisms; by direct interaction of ID2 and ID4 with OLIG1 and OLIG2, and indirectly by sequestration of E2A proteins by all the IDs.

Olig genes can partially rescue oligodendrogenesis in the presence of BMP4

In order to ascertain whether OLIG proteins are capable of reversing the effects of BMP4, we overexpressed *Olig1* and *Olig2* alone or along with *Id4* or *Id4*-RNAi (Fig. 8A-C). In the

absence of BMP4, overexpression of Olig genes resulted in a significant increase in numbers of CNPase⁺ and MBP⁺ OLs along with a decrease in the number of GFAP⁺ astrocytes (Fig. 8A,B). In the presence of BMP4, *Olig1* and *Olig2* resulted in much smaller but still significant increases in CNPase⁺ and MBP⁺ OLs ($P < 0.05$) along with a decrease in the number of GFAP⁺ astrocytes ($P < 0.05$) compared with BMP4-treated cells infected with the control GFP virus (Fig. 8B) ($P < 0.05$ by Anova). This suggests that *Olig1* and *Olig2* can partially reverse the effects of BMP4 on lineage commitment. Overexpression of Olig genes along with *Id4*-RNAi almost completely blocked the effects of BMP4 on lineage commitment (Fig. 8C).

Discussion

Neuroectodermal cells located along the entire length of the rostrocaudal axis of the neural tube have the potential to generate OLs (Hardy and Friedrich, 1996). Nevertheless, OLs in the developing nervous system originate from discrete domains in the ventral telencephalon and spinal cord in chick (Cameron-Curry and Le Douarin, 1995; Ono et al., 1995), rodents (Liu et al., 2002; Noll and Miller, 1993; Qi et al., 2002; Sussman et al., 2000; Timsit et al., 1995; Warf et al., 1991; Yu et al., 1994) and humans (Hajhosseini et al., 1996). The dorsal spinal cord has cells capable of generating OLs in vitro (Cameron-Curry and Le Douarin, 1995; Sussman et al., 2000; Trousse et al., 1995); when dorsal spinal cord explants are co-cultivated with notochord or floor plate tissue, numerous OLs are produced. However, the explants cultured alone fail to produce OLs, indicating the presence of a dorsal inhibitory factor (Orentas and Miller, 1996; Poncet et al., 1996;

Trousse et al., 1995; Wada et al., 2000; Yamada et al., 1991). Collectively these studies suggest that the development of OLs is under the control both of ventral inductive signals and dorsal inhibitory signals.

BMP signaling inhibits oligodendrogenesis in vitro and in vivo

BMPs secreted from the roof plate provide dorsalizing signals during development and inhibit the formation of

ventral cell types (Liem et al., 1997; Liem et al., 1995) whereas Shh produced by the notochord and floor plate provides ventralizing signals promoting the development of OLs (Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996; Trousse et al., 1995). Several studies have suggested that the development of OL depends on the balance between the dorsalizing BMP signaling and ventralizing Shh signaling (Liem et al., 2000; Mekki-Dauriac et al., 2002; Patten and Placzek, 2002).

BMP signaling inhibits oligodendrogenesis from cultured progenitor cells and enhances astrogliogenesis (Gross et al., 1996; Li et al., 1998; Mabie et al., 1999; Mabie et al., 1997; Mehler et al., 1995; Grinspan et al., 2000; Zhu et al., 2000). Further, overexpression of BMP4 in transgenic mice under the control of neuron-specific enolase promoter results in a significant decrease in OL numbers concurrent with an increase in astrocytes (Gomes et al., 2003). Conversely, overexpression of noggin under the control of the same promoter increases the number of OLs and decreases astrocytes (J.A.K., unpublished). Finally, ablation of the dorsal spinal cord in chick embryos or inactivation of BMP signaling by noggin in vivo results in the appearance of OLPs dorsal to their normal domain (Mekki-Dauriac et al., 2002). Taken together, these studies provide strong evidence that BMP signaling provides the inhibitory signal that restricts the foci of oligodendrogenesis in vivo. Thus, ventral Shh signaling induces expression of OL lineage species whereas dorsal BMP signaling inhibits oligodendroglialogenesis.

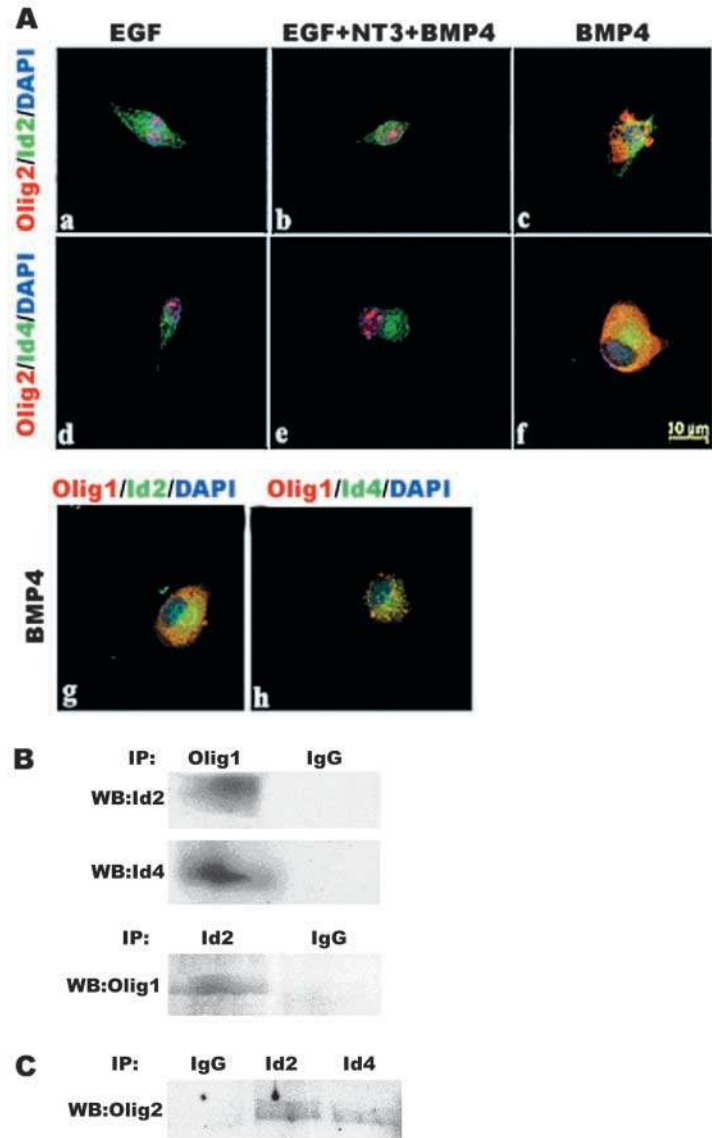


Fig. 6. OLIG proteins co-localize and interact with ID2 and ID4 in neural progenitor cells. (A) E17 neural progenitor cells were grown for 7 days in the presence of 10 ng/ml EGF (parts a,d), 3 days with 10 ng/ml PDGF-AA+NT3+EGF (parts b,e) and 12 hours with 20 ng/ml BMP4 (parts c,f-h). Cells were plated and stained for OLIG1 and ID2 (part g), OLIG1 and ID4 (part h), OLIG2 and ID2 (parts a-c) and OLIG2 and ID4 (parts d-f). Nuclei were counterstained with DAPI. In the absence of BMP4, both OLIG1 and OLIG2 were localized primarily in the nucleus. However, after BMP4 treatment both OLIG1 and OLIG2 were colocalized with ID2 and ID4 in the cytoplasm. (B,C) Co-immunoprecipitations were done with protein lysates from cultured E17 neural progenitor cells treated with BMP4 for 12 hours. OLIG1 (B) and OLIG2 (C) interact with both ID2 and ID4.

ID proteins act as effectors of BMP4 signaling

Which intracellular factors mediate the effects of BMP signaling on lineage commitment? BMP4 treatment of cultured neural progenitor cells increases expression of all four ID proteins (Fig. 2). BMPs also induce Id expression in several other cell types, including osteoblasts (Ogata et al., 1993) and embryonic stem cells (Hollnagel et al., 1999). Moreover, the *Id1* promoter is activated by BMPs in a smad-dependent manner (Korchynskiy and ten Dijke, 2002; Lopez-Rovira et al., 2002) suggesting that the *Ids* are direct targets of BMP signaling (Miyazono and Miyazawa, 2002). The Id family includes four structurally related proteins ID1-4 which act as dominant negative antagonists of bHLH transcription factors in various cell lineages (Benezra et al., 1990; Riechmann et al., 1994; Sun et al., 1991). Each ID protein has a distinct pattern of expression in the developing nervous system with characteristic spatial and temporal patterns in restricted cell types. For example, ID1 and ID3 are detected in dividing neuroblasts, whereas ID2 and ID4 are expressed in relatively mature neurons, and ID2 and ID3 are absent in O4⁺ OLs (Jen et al., 1996; Jen et al., 1997; Tzeng and de Vellis, 1998). This suggests that the cellular specificity of the Id gene family may play a critical role in lineage commitment. Expression of *Id2* and *Id4* declines progressively as OLPs undergo maturation, and overexpression of *Id2* and *Id4* in OLPs inhibits OL differentiation, whereas their absence induces premature differentiation in vitro (Kondo and Raff, 2000; Wang et al., 2001). Because ID2 and ID4 levels decline when

Table 1. OLIG1 and OLIG2 directly bind to ID2 and ID4

Constructs	Number of colonies		
	pBT- <i>OLIG</i> + pTRG- <i>ID</i> (test)	pBT- <i>OLIG</i> + pTRG (negative control)	pBT + pTRG- <i>ID</i> (negative control)
<i>OLIG1</i> + <i>ID2</i>	79	5	3
<i>OLIG1</i> + <i>ID4</i>	60	2	0
<i>OLIG2</i> + <i>ID2</i>	97	4	5
<i>OLIG2</i> + <i>ID4</i>	83	1	6

Bacterial two-hybrid analyses was carried out to detect possible direct binding using bait vectors pBT-*OLIG1* or pBT-*OLIG2* and target vectors pTRG-*ID2* or pTRG-*ID4*. The negligible number of colonies in the negative control plates and the large number of colonies in the test plates indicate a positive interaction of OLIG proteins with ID2 and ID4. No interactions were detected with ID1 or ID3 (not shown).

OLPs are induced to differentiate by withdrawal of PDGF, it was hypothesized that the IDs enhance the rate of proliferation and thus inhibit OL differentiation when overexpressed in OLPs (Kondo and Raff, 2000; Wang et al., 2001). In this study, we found that overexpressing *Id2* and *Id4* in cultured progenitor cells not only inhibits OL fate specification but also increases astrocytic differentiation (Fig. 3), similar to that observed with BMP4 treatment. Further, inhibition of *Id4* expression reversed the phenotype seen with BMP4 exposure (Fig. 3). Because BMPs induce the ID proteins, we can conclude that the IDs mediate effects of BMP signaling on glial lineage commitment.

Molecular mechanism of inhibition of oligodendrogenesis

How does ID expression inhibit oligodendroglial lineage commitment? The IDs are HLH factors, which lack a DNA binding basic domain. Thus, when they dimerize with other bHLH transcription factors, they inhibit DNA binding and prevent transcription of downstream targets (Christy et al., 1991; Massari and Murre, 2000). They typically produce their effects by binding to the ubiquitously expressed class A bHLH factors such as E2A, HEB and E2-2 proteins (Benezra et al., 1990; Jogi et al., 2002; Sun et al., 1991). However, we found that all the ID proteins complexed with E2A proteins but only ID4 and ID2 inhibited oligodendroglial lineage commitment. This suggested that ID4 and ID2 must exert their effects by binding to other factors. ID proteins also bind directly to the tissue-specific class B bHLH factors MyoD and Myf5 that are involved in muscle differentiation (Langlands et al., 1997). This suggested that the ID4 and ID2 proteins might exert their effects on OL lineage commitment by binding to other class B bHLH transcription factors.

Olig1 and *Olig2* are class B bHLH transcription factors that are important for oligodendrogenesis (Lu et al., 2000; Zhou et al., 2000) and spinal motor neuron development (Lu et al., 2002; Mizuguchi et al., 2001; Novitsch et al., 2001; Park et al., 2002; Zhou and Anderson, 2002). *Olig2* is expressed earlier than *Olig1* and both bHLH factors are detectable in OLPs in the vertebrate CNS (Zhou et al., 2000). Ectopic expression of *Olig1* in vivo and in vitro induces the formation of OL

precursors (Lu et al., 2001; Lu et al., 2000), whereas *Olig2* expression in vivo causes ectopic expression of *Sox10* (Zhou et al., 2000). Sox10 contains a DNA binding domain of the high mobility group (HMG) and is predominantly expressed in OLs in the CNS (Stolt et al., 2002). It is also known to directly bind to and activate the myelin basic protein promoter, thus suggesting a role in differentiation of OLs. In the spinal cord, the glial precursor domain defined by the expression of *Pdgfra/Olig1/Olig2* has been mapped to the ventral border of the *Pax6* expression domain but dorsal to the *Nkx2.2* domain (Lu et al., 2000; Zhou et al., 2000). Our results show that ID4 and ID2 bind to OLIG1 and OLIG2 (Figs 5, 6) and thus inhibit the OLIGs from binding to DNA. Because OLs do not develop in the absence of OLIG1/2 function (Lu et al., 2002; Zhou and Anderson, 2002), sequestration of the OLIG proteins by ID4 and ID2 would be expected to inhibit oligodendroglialogenesis (Fig. 9). We also found that BMP4 treatment resulted in cytoplasmic rather than nuclear localization of OLIG1 and OLIG2 (Fig. 6A), suggesting that binding to ID proteins prevented the translocation of OLIG proteins to the nucleus. ID proteins are expressed in the cytoplasm of progenitor cells even in the absence of BMP signaling (Figs 2, 6), and BMP4 treatment did not alter the intracellular localization of the ID proteins. Both western analyses and quantitative RT-PCR demonstrated large increases in expression of the IDs in response to BMP4 (Fig. 2), although the increase in ID proteins after BMP4 treatment was not clearly demonstrated by non-quantitative immunocytochemistry (Fig. 6). This suggests that ID4 and ID2 must reach critical levels in the cytoplasm to effectively bind and sequester the OLIG proteins, thereby preventing their translocation

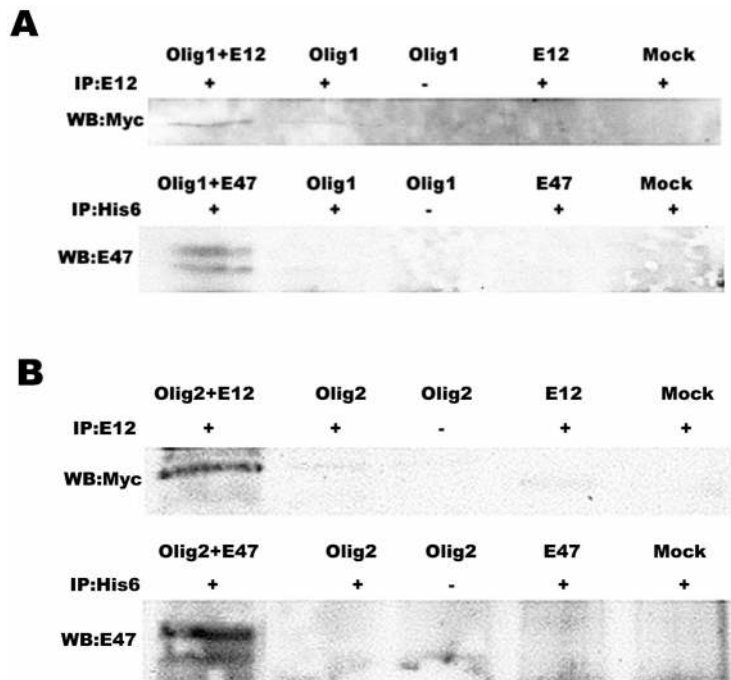


Fig. 7. OLIG1 and OLIG2 interact with E2A proteins E12 and E47. Co-immunoprecipitations were done with protein lysates from HEK293T cells transfected with *Olig1/2*-myc-his6 and E12 or E47. OLIG proteins were pulled down with his6 antibody and immunoblotted with myc antibody, whereas specific E2A antibodies were used to pull down or immunoblot E2A proteins. OLIG1 (A) and OLIG2 (B) interact with both E12 and E47.

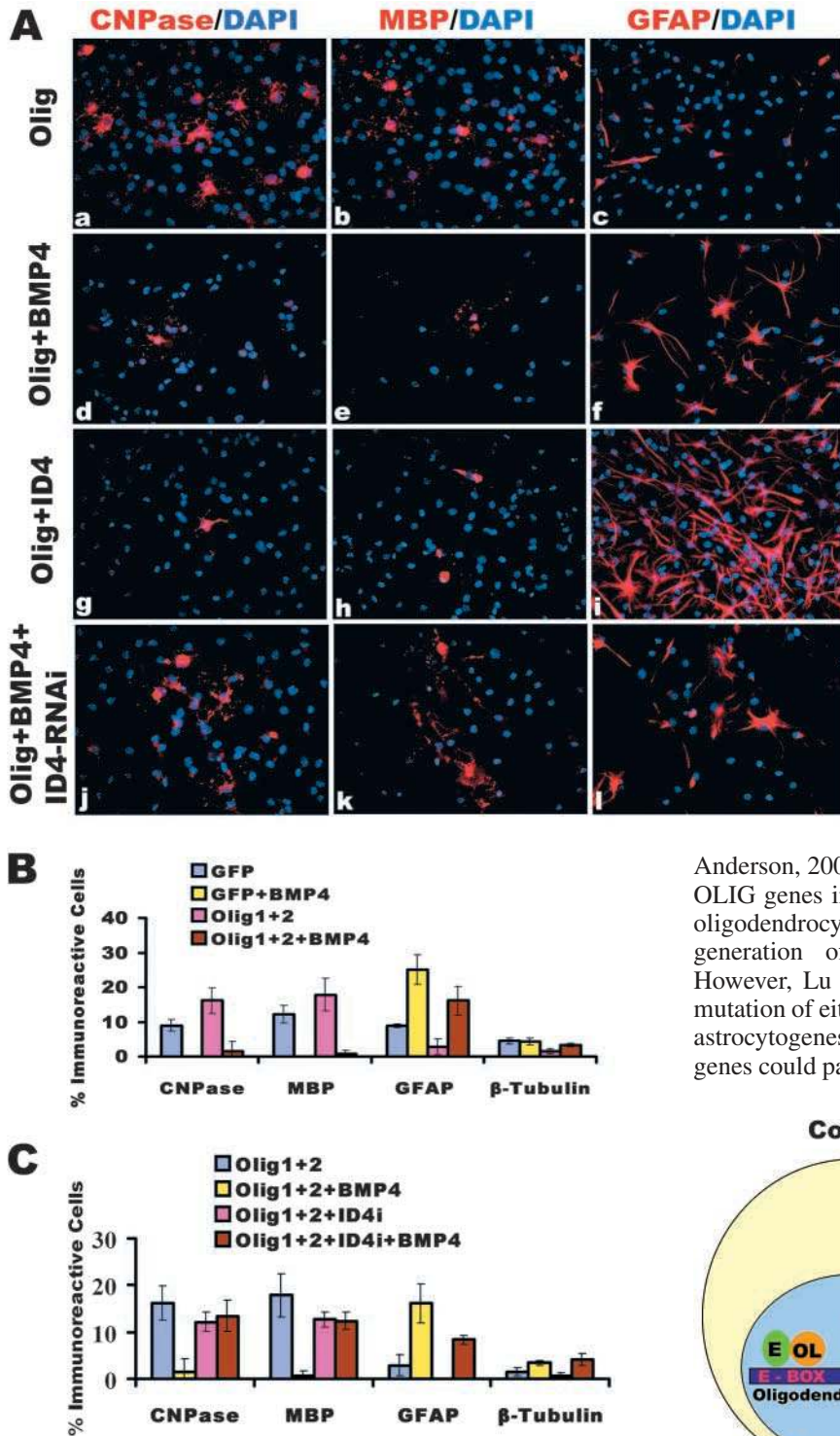


Fig. 8. OLIG proteins partially overcome BMP4 effects. (A) Cultured E17 progenitor cells were infected for 2 days with lentivirus containing *Olig1* or *Olig2* (parts a-l) along with *Id4* (parts g-i) or *Id4*-RNAi (parts j-l). Cells were treated with 20 ng/ml BMP4 in parts d-f and parts j-l. Immunofluorescence was done for CNPase (parts a,d,g,j), MBP (parts b,e,h,k) and GFAP (parts c,f,i,l). DAPI was used to stain the nuclei. (B,C) Quantification for CNPase, GFAP and β -tubulinIII shows that OLIG proteins can partially rescue the effects of BMP4 treatment or ID4 overexpression on oligodendroglia. The rescue is complete with *Id4*-RNAi (C). Scale bars represent means \pm s.e.m. of four independent experiments. ANOVA was done to determine significance ($P < 0.05$).

to the nucleus. OLPs are known to express the E2A proteins, E12 and E47 as well as OLIG proteins (Sussman et al., 2002), and our results demonstrate that the E2A proteins are binding partners for the OLIG proteins (Fig. 7). Because the ID proteins also bind to E2A proteins, this suggests another mechanism by which the ID proteins may inhibit OLIG function.

After 8 hours of BMP4 treatment, Olig expression gradually declined in cultured progenitor cells co-incident with respecification of lineages; this suggests an indirect effect of BMP signaling on Olig gene expression, although a direct effect cannot be excluded. This conclusion is consistent with the findings of Mekki-Dauriac et al. that no OLIG2-expressing cells were detectable in proximity to an implanted source of BMP4 after 48 hours. Mekki-Dauriac et al. did not detect an obvious increase in astrocytes that would indicate respecification of lineage, but they did not do detailed cell counts to address this point. However, a recent paper by Gomes et al. (Gomes et al., 2003) demonstrates that overexpression of BMP4 in mouse brain does lead to an increase in astrocytes coupled with a decrease in oligodendroglia. Further, studies by Zhou and Anderson (Zhou and

Anderson, 2002) have shown that double null mutation of both OLIG genes in mice results in inhibition of the generation of oligodendrocytes in vivo coupled with an apparently enhanced generation of astrocytes, consistent with our findings. However, Lu et al. (Lu et al., 2002) have reported that null mutation of either OLIG gene alone is not sufficient to increase astrocytogenesis in the spinal cord, possibly because the Olig genes could partially complement the functions of one another.

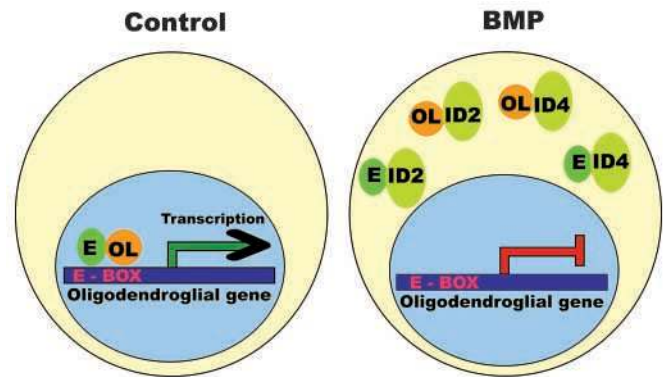


Fig. 9. Model of the role of ID proteins in mediating effects of BMP signaling. OLIG proteins bind to promoter regions after heterodimerizing with the E2A proteins, resulting in transcription of their downstream targets. In the presence of BMP4, all four ID proteins are induced, resulting in sequestration of OLIG 1/2 by ID2 and ID4 and of E2A proteins by all the IDs in the cytoplasm of progenitor cells. This inhibits OLIG binding to promoters of target genes, resulting in inhibition of OL development and enhancement of commitment to the astrocytic fate.

It is interesting that overexpression of ID4 promoted astrocyte lineage commitment analogous to the effects of BMP signalling, whereas *Id4*-RNAi inhibited the generation of astrocytes. There are two possible interpretations of this finding. First, it is possible that ID4 exerts actions that directly foster the generation of astrocytes. Alternatively, astroglialogenesis may simply be fostered by the inhibition of commitment to the alternate OL lineage. BMPs and LIF act synergistically to promote astrocytic differentiation of neural progenitor cells (Nakashima et al., 1999). They induce the transcription factors *smad1* and *STAT3* which form a complex with p300 in the nucleus, resulting in direct activation of the GFAP promoter. *Olig2* represses the astrocyte-specific GFAP promoter by interacting with p300 and thereby abolishes the complex between *STAT3*, *smad1* and p300 (Fukuda et al., 2004). The dominant negative effects of the ID proteins on OLIG functions would thus derepress GFAP expression and enhance astrocyte specification as well as inhibit oligodendroglial lineage commitment (Figs 3, 8). In *Olig1/2* double-mutant mice, there is a complete failure of OL development in all areas of the brain and instead there is a small increase in astrocytogenesis (Zhou and Anderson, 2002). This suggests that repression of OL development may be sufficient to cause astroglialogenesis, consistent with the findings of Fukuda et al. (Fukuda et al., 2004) as well as our own findings.

In summary, BMP signaling regulates glial lineage commitment by inducing expression of the ID proteins, particularly ID4, which complex with both OLIG proteins and with the E2A proteins which are OLIG binding partners (Fig. 9). This inhibits the generation of OLs in regions where BMP signaling predominates, such as the dorsal spinal cord, and may enhance the generation of astrocytes.

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