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on Proliferation and Differentiation of Embryonic Neural Progenitor Cells

Gaofa Zhu,[1] Mark F. Mehler, Jie Zhao, Shau Yu Yung, and John A. Kessler

Department of Neurology and Department of Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Although Sonic Hedgehog (Shh) plays a critical role in brain development, its actions on neural progenitor cell proliferation and differentiation have not been clearly defined. Transcripts for the putative Shh-receptor genes *patched* (Ptc) and *smoothened* (Smo) are expressed by embryonic, postnatal, and adult progenitor cells, suggesting that Shh can act directly on these cells. The recombinant human amino-terminal fragment of Shh protein (Shh-N) alone did not support the survival of cultured progenitor cells, but treatment with Shh-N in the presence of bFGF increased progenitor cell proliferation. Furthermore, treatment of embryonic rat progenitor cells propagated either in primary culture or after mitogen expansion significantly increased the proportions of both β-tubulin- (neuronal marker) and O4- (oligodendroglial marker) immunoreactive cells and reduced the proportion of nestin- (uncommitted neural progenitor cell marker) immunoreactive cells. By contrast Shh-N had no effect on the elaboration of GFAP- (astroglial marker) immunoreactive cells. Cotreatment with Shh-N and bone morphogenetic protein-2 (BMP2) inhibited the anti-proliferative, astroglial-inductive, and oligodendroglial-suppressive effects of BMP2. Our observations suggest that Shh-N selectively promotes the elaboration of both neuronal and oligodendroglial lineage species and inhibits the effects of BMP2 on progenitor cell proliferation and astroglial differentiation. **© 1999 Academic Press**

Key Words: **Sonic Hedgehog; bone morphogenetic protein; patched; smoothened; neural lineage development; proliferation.**

INTRODUCTION

Sonic hedgehog (Shh) is a member of the *hedgehog* (hh) multigene family that encodes signaling proteins involved in induction and patterning processes in vertebrate and invertebrate embryos (for reviews see Fietz *et al.*, 1994; Smith, 1994; Bumcrot and McMahon, 1995; Goodrich and Scott, 1998). The early expression of Shh is limited to the notochord, the floor plate and its anterior extension in the brain, and the zone of polarizing activity (ZPA) in the posterior mesenchyme of the limb bud (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Chang *et al.*, 1994; Roelink *et al.*, 1994; Bitgood and McMahon, 1995). Shh appears to be involved in the induction of neuronal phenotypes in the brain (Roelink *et al.*, 1994, 1995; Hynes *et al.*, 1995; Wang *et al.*, 1995; Ericson *et al.*, 1995, 1996, 1997; Ye *et al.*, 1998) and in the induction of oligodendrocyte lineage commitment in the spinal cord (Orentas and Miller, 1996; Pringle *et al.*, 1996). Targeted disruption of the Shh gene leads to the absence of ventral cell types within the neural tube, absence of the spinal column and most ribs, cyclopia, and absence of distal limb structures (Chiang *et al.*, 1996). Interactions between Shh and members of the bone morphogenetic protein (BMP) gene family are important for the specification of dorsal and intermediate dorsoventral cell types (for reviews see Roelink, 1996; Tanabe and Jessell, 1996; Goodrich and Scott, 1998), and Shh inhibits BMP signaling, in part by inducing the endogenous BMP inhibitor, noggin (Hirsinger *et al.*, 1997). Additional interactions between Shh and other signaling molecules including Wnt, FGF4, and FGF8 are critical for specifying

[1] To whom correspondence should be addressed. Fax: (718) 430-8992. E-mail: gzhu@aecom.yu.edu.

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alternate cellular phenotypes in the brain (Ye *et al.,* 1998) and for patterning of the dorsal compartment of the somite (Marcelle *et al.,* 1997).

In addition to its effects on axial patterning and cellular differentiation, Shh appears to regulate cellular proliferation. The factor is a potent mitogen for cultured retinal progenitor cells (Jensen and Wallace, 1997; Levine *et al.,* 1997), cerebellar granule cell precursors (Wechsler-Reya and Scott, 1999), and skeletal muscle cells (Duprez *et al.,* 1998), and overexpression of the Shh gene leads to basal cell carcinoma (Fan *et al.,* 1997; Oro *et al.,* 1997). Disruption of the gene encoding the Shh-binding protein *Patched* (Ptc) leads to meduloblastoma and other primitive neuroectodermal tumors (Goodrich *et al.,* 1997; Wolter *et al.,* 1997). To activate target genes, the N-terminal signaling domain of Shh (Shh-N) binds to Ptc, which is complexed with *Smoothened* (Smo), to counteract the inhibition by Ptc of constitutive signaling activity mediated by Smo (for reviews see Alcedo and Noll, 1997; Tabin and McMahon, 1997).

Although cumulative evidence indicates that Shh plays a critical role in neural development, the cellular actions of Shh on neural stem/progenitor cells have not been clearly defined. Further, the profound disruption of axial patterning and early neural development in mice lacking functional Shh (Chiang *et al.,* 1996) has precluded evaluation of the role of the factor in later embryogenesis and postnatal development. Therefore, in these studies we examined the effects of Shh on survival, proliferation, and differentiation of embryonic neural progenitor cells. Moreover, since our laboratory has previously shown that BMPs suppress oligodendroglial differentiation and promote the elaboration of the astroglial lineages (Gross *et al.,* 1996; Mabie *et al.,* 1997; Zhu *et al.,* 1999), experiments were also performed to examine the cellular consequences of interactions between Shh and BMP2 signaling. We find that Shh selectively promotes the elaboration of both neuronal and oligodendroglial lineage species and inhibits the effects of BMP2 on progenitor cell proliferation and astroglial differentiation.

MATERIALS AND METHODS

Animals. Timed-pregnant embryonic (E), postnatal (PN), and adult (250–350 g, 6–18 months old) Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY).

Growth factors. Recombinant human basic fibroblast growth factor (bFGF) was purchased from Collaborative Biomedical Products (Bedford, MA), recombinant human BMP2 was generously supplied by Genetics Institute (Cambridge, MA), and recombinant human amino-terminal fragment of Shh protein (Shh-N) was generously provided by Biogen and Ontogeny, Inc. (Cambridge, MA, Batch KW3367-12JK).

Generation of progenitor cell neurospheres and culture of dissociated cells. The forebrains of E12.5 rat embryos and the striata of E15.5 embryos were dissociated using fire-polished pasteur pipettes. The subventricular zones (SVZs) of E18.5 and PN2 animals were dissociated by initial incubation with 0.025% trypsin (Sigma, St. Louis, MO) for 15 min followed by repeated trituration. The subependymal regions of the lateral ventricles of PN15 and adult rats were dissociated and processed as previously described (Reynolds and Weiss, 1992). Dissociated cells (4×10^5/dish) were seeded onto 60-mm^2 uncoated culture dishes (Falcon, Franklin Lakes, NJ) in serum-free medium (SFM) in the presence of bFGF (10 ng/ml) + heparin (2 μg/ml) (Sigma, 178 units/mg) and were cultured for 6 days to generate proliferative progenitor neurospheres at the stage of maximal size and viability. The SFM consisted of DMEM/F12 (Gibco BRL, Grand Island, NY) with nutrient additives (0.01% BSA, fraction 5; 30 nM selenium; 15 nM triiodo-L-tryronine (T3); 40 nM biotin; 25 mM apo-transferrin; 1 nM hydrocortisone; 25 μg/ml insulin (all reagents were from Sigma), 1:100 DMEM nonessential amino acids, and 1:100 antibiotic/antimycotic (both reagents from Gibco)).

After 6 days in culture, bFGF-generated neurospheres were dissociated by initial incubation with 0.025% trypsin for 15 min followed by repeated trituration. A total of 4×10^4 dissociated cells per well were plated onto poly-D-lysine-coated (PDL, Sigma, 20 μg/ml for 1 h) coverslips (12 mm; Carolina Biologicals, Burlington, NC) within 24-well multiwell culture plates and were propagated in SFM with or without bFGF, Shh-N, and/or BMP2.

For primary E15.5 striatal cell cultures, E15.5 striatum was mechanically dissociated using fire-polished pasteur pipettes and 4×10^4 cells per well were plated onto PDL-coated coverslips within 24-well multiwell culture plates in SFM with or without bFGF and/or Shh-N.

Immunocytochemistry. Cells were fixed with ice-cold methanol and processed for standard indirect immunofluorescence staining. Fixed coverslips were washed in phosphate-buffered saline (PBS) and incubated with primary antibodies using appropriate blocking serum (5% normal goat serum) at 4°C overnight or at room temperature for 2–3 h. The following antibodies were used: β-tubulin (type III) (mouse IgG2b, 1:400, Sigma), nestin (mouse IgG1, 1:1000, Pharmingen, San Diego, CA), GFAP (mouse IgG1, 1:400, Sigma). For O4 staining, cultured cells were incubated for 30 min at 4°C in O4 hybridoma culture supernatant (the O4 hybridoma line was a gift from Dr. S. Pfeiffer, University of Connecticut). Cells were then washed once with PBS and fixed as described above. Following washing in PBS three times, secondary antibodies were applied. The appropriate isotype-specific secondary antibodies consisted of either TRITC- or FITC-conjugated preparations (1:200, Southern Biotechnologies, Birmingham, AL). Following repeat washings (×2) in PBS, coverslips were counterstained with Hoechst 33342 (2 μg/ml, Molecular Probes, Eugene, OR) to visualize nuclei and to determine total cell numbers. After being washed with water (×2), coverslips were mounted using antifade reagent (Molecular Probes) and photographed using an Olympus BX50 epifluorescence microscope. The cellular counts for each experimental condition were obtained by counting 10 alternate fields of view of each coverslip from at least five independent culture wells; the results of each experimental condition were verified in a minimum of three independent experiments.

Live/Dead staining. The Live/Dead viability kit was purchased from Molecular Probes, Inc. The principle of this assay is that membrane-permeant calcein AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised dead cells with red fluorescence. Staining was performed using the manufacturer's protocol to determine the viability of cultured cells. The cellular counts were performed as above.

BrdU incorporation assay. To label cells in the S-phase of the cell cycle, BrdU (10 μM) (Boehringer Mannheim) was added to the culture medium for 16 h. The medium was subsequently removed

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and the cells were washed in PBS. The cells were then fixed in 100% cold methanol for 15 min, rehydrated in PBS, incubated in 2 M HCl for 45 min at room temperature to denature the DNA, again washed in PBS, neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5) for 15 min, and washed in PBS. Cellular preparations were incubated with anti-BrdU antibody (1:400, Vector Laboratories, Burlingame, CA) with 5% goat serum overnight at 4°C, followed by incubating with FITC-conjugated secondary antibody (1:200, Southern Biotechnologies) for 1 h at room temperature. After being washed in PBS (×2), coverslips were counterstained with Hoechst 33342 (2 μg/ml, Molecular Probes) to visualize nuclei and to determine total cell numbers. After being washed twice with water, coverslips were mounted using antifade reagent (Molecular Probes) and photographed using an Olympus BX50 epifluorescence microscope. The cellular counts were performed as above.

In situ hybridization. E12.5, E15.5, and E18.5 rat embryos were frozen in cold (−25°C) 2-methylbutane (Sigma) and stored at −70°C before cryostat sectioning. Sections (14 μm) were cut. *In situ* hybridization was performed by postfixing sections in 0.1 M sodium phosphate-buffered 4% paraformaldehyde, pH 7.4, for 30 min, rinsing in PBS for 1 min, rinsing in 2× SSC for 1 min, acetylation with 0.5% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min, rinsing again in 2× SSC and then in PBS, and finally dehydrating in a graded series of ethanol washes. The slides were prehybridized in 2× SSC and 50% formamide at 50°C for 2 h and hybridized using hybridization buffer containing 2×10^4 cpm/μl cRNA probe (hybridization buffer, 0.75 M NaCl, 50% formamide, 1× Denhardt's solution, 10% dextran sulfate, 30 mm DTT, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 μg/ml salmon sperm DNA, and 0.5 mg/ml yeast tRNA) at 50°C for 16 h. Both antisense and sense riboprobes were transcribed from mouse Ptc and Smo cDNAs (Ontogeny) with a Promega (Madison, WI) kit following the manufacturer's instructions. Slides were then washed twice in 2× SSC for 2 min; in 2× SSC, 50% formamide, and 0.1% β-mercaptoethanol (BME) at 50°C for 1 h; in 20 μg/ml RNase A at 37°C for 30 min; in 0.5 M NaCl and 10 mM Tris–HCl, pH 8.0; in 2× SSC, 50% formamide, and 0.1% BME at 58°C for 30 min; and in 0.1× SSC and 0.1% BME at 63°C for 30 min, with final dehydration. The sections were then exposed to X-ray film for 4 or 5 days to obtain autoradiograms, dipped in emulsion, and exposed for 3–4 weeks before development. Use of the sense riboprobe confirmed the specificity of labeling.

RT-PCR. Total RNA was isolated from bFGF-generated neurospheres using Tri-reagent (MRC, Cincinnati, OH) as prescribed by the manufacturer. Genomic DNA was removed from the RNA by incubation with 0.5 U of RNase-free DNase I (Ambion, Austin, TX) at room temperature for 15 min following by heating at 75°C for 5 min to inactivate the DNase. Reverse transcription was performed using random hexameric primers and MuLV reverse transcriptase (RT) (Promega) as suggested by the manufacturer. Negative control RT reactions were performed as above but without the addition of reverse transcriptase. *Taq* DNA polymerase (Gibco BRL) was used to perform the PCR as follows: for Ptc, 30 cycles of 95°C for 35 s, 62°C for 25 s, and 72°C for 20 s following by 72°C for 10 min. The Ptc-specific primers were CATTGGCAGGAGGAGTTGATTGTGG (forward primer) and AGCACCTTTTGAGTGGAGTTTGGGGG (reverse primer). For Smo, 30 cycles of 95°C for 35 s, 60°C for 60 s, and 72°C for 60 s followed by 72°C for 10 min. The Smo-specific primers were GTAGTGTGGTTCGTGGTCCTC (forward) and GGTTGGCTTGTTCTTCTGGTG (reverse). For Shh, 94°C for 3 min, 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s followed by 72°C for 10 min. The Shh-specific primers were CGGCCGATATGAAGGGAAGA (forward) and CGGAGTTCTCTGCTTTCACA (reverse). For actin, 30 cycles of 95°C for 35 s, 54°C for 25 s, and 72°C for 20 s followed by 72°C for 10 min. The actin-specific primers were GGCTCCGGTATGTGC (forward primer) and GGGGTACTTCAGGGT (reverse primer). The PCR mixtures were then fractionated by agarose electrophoresis. PCR products were sequenced to verify the identities of the cDNAs.

RESULTS

Neural Progenitor Cells Express Ptc and Smo but Not Shh

To determine whether neural progenitor cells express mRNAs encoding Shh and its putative receptor-associated genes Ptc and Smo, proliferative progenitor neurospheres were generated from the VZ or SVZ regions of animals of different ages ranging from E12.5 to adult. After 6 days *in vitro* (DIV), total RNA was isolated from the neurospheres and RT-PCR was performed. Amplification products were not observed when reverse transcriptase was omitted from the reactions, suggesting that there was no genomic DNA contaminating the samples. Ptc and Smo mRNAs were expressed by progenitor cells at all ages with the highest levels of expression in embryonic cells (Fig. 1A). The identity of the PCR products was verified by sequencing. By contrast, these cells did not express the ligand, Shh, at any age (Fig. 1B). To determine whether Ptc and Smo are expressed by ventricular zone progenitor cells *in vivo, in situ* hybridization analyses were performed. The results (Fig. 2) revealed that mRNAs encoding both Ptc and Smo are widely expressed in the developing brain. Intense labeling for Ptc mRNA is seen within the forebrain, midbrain, and hindbrain of E12.5 (Fig. 2A) and E15.5 (Fig. 2B) embryos and also within the SVZ and tegmentum of E18.5 embryos (Fig. 2C). Smo mRNA (Figs. 2E–2G) displayed a pattern of expression similar to that of Ptc mRNA with additional transcript expression in the olfactory bulb of E18.5 embryos (Fig. 2G).

Shh-N Increases the Proliferation of Embryonic Forebrain Progenitor Cells but Is Not a Survival Factor

To examine the possible effects of Shh-N on progenitor cell survival and proliferation, bFGF-generated progenitor cell neurospheres from E12.5 forebrain were dissociated and plated on PDL-coated coverslips in the absence or presence of bFGF (10 ng/ml) and/or Shh-N (5 nM). Live/Dead staining performed 2 days later (Figs. 3A–3D) indicated that the progenitor cells failed to survive in the presence of Shh-N alone (Fig. 3B). However, they survived well in the presence of bFGF (Fig. 3C), and the addition of Shh-N along with bFGF (Figs. 3D and 3E) further increased the number of live cells ($P < 0.01$). The effects of Shh-N could reflect an increase in progenitor cell survival and/or proliferation. However, treatment with Shh-N did not reduce the number of dead cells (Fig. 3E) mitigating against a survival effect and

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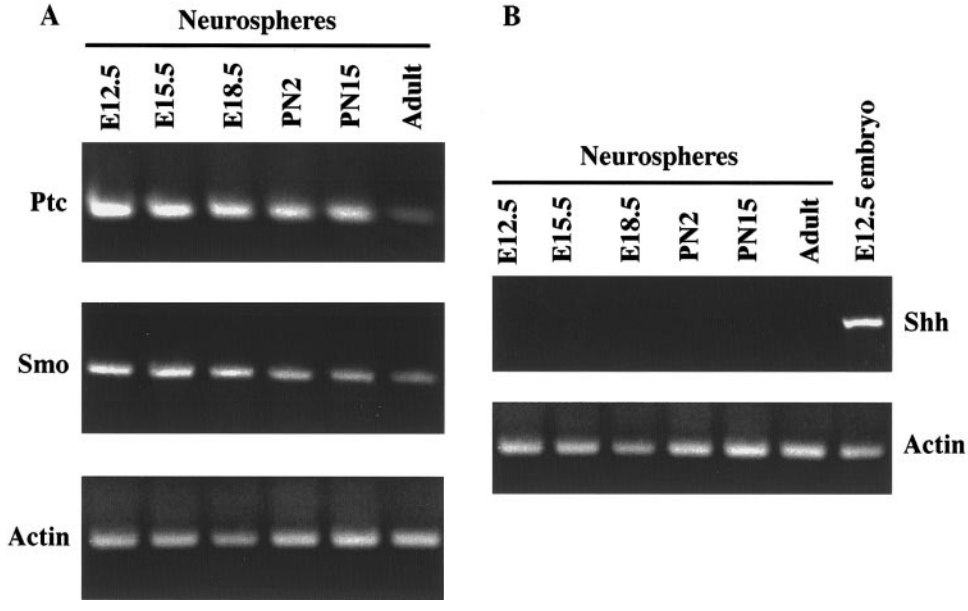

FIG. 1. Neural progenitor cells express the Shh receptor-associated genes, Ptc and Smo, but do not express Shh itself. Dissociated cells from embryonic E12.5 (forebrain), E15.5 (striatum), E18.5 and PN2 (SVZ), PN15 and adult (subependymal region of the lateral ventricle) rat brains were cultured in SFM in the present of bFGF (10 ng/ml) + heparin (2 μg/ml) to generate neurospheres. After 6 days, total RNA was isolated from these neurospheres and RT-PCR was performed (see Materials and Methods for details) to examine the expression of Ptc, Smo (A), and Shh (B) mRNAs in bFGF-generated neurospheres. RT-PCR for actin was used as a normalization control. Amplification products were not observed when reverse transcriptase was omitted from the reaction mixtures.

suggesting an effect on proliferation. To more rigorously define the effects of Shh-N on cellular proliferation, neurospheres generated from E12.5 forebrain were dissociated and plated in the presence of bFGF (10 ng/ml) and different doses of Shh-N (1, 1.25, 2.5, 5, 10, and 20 nM). After 2 days in culture, BrdU (10 μM) was added for 16 h, followed by BrdU immunostaining. Addition of Shh-N to bFGF treated cultures significantly increased the proportion of proliferating cells (BrdU-immunoreactive cells) (Fig. 4) in a dose-dependent fashion (Fig. 4C). Half-maximal stimulation of proliferation occurred at a dose of 2.5 nM, and effects saturated at a dose of 5 nM. Thus, while Shh-N alone cannot support the survival of neural progenitor cells, it is a mitogen for cells maintained in the presence of bFGF.

Shh-N Enhances Neuronal and Oligodendroglial Differentiation of Both Primary and Mitogen-Expanded Cultures of Embryonic Progenitor Cells

To define the effects of Shh-N on the differentiation of neural progenitor cells, E12.5 forebrain-derived neurospheres were dissociated and plated in the presence of different doses of Shh-N (0, 1.25, 2.5, 5, 10, and 20 nM). bFGF (10 ng/ml) was also added initially to maintain progenitor cell survival but was removed after 2 days to allow the cells to differentiate independent of the ongoing effects of bFGF; removal of bFGF at this time did not alter subsequent cell survival (data not shown). After 6 DIV, immunofluorescence staining was performed using nestin, β-tubulin, O4, and GFAP antibodies (Figs. 5A–5D). Both untreated and Shh-N treated progenitor cells gave rise to neuronal, astroglial, and oligodendroglial lineage species. However, treatment with Shh-N significantly increased the proportion of both β-tubulin- and O4-immunoreactive cells and reduced the proportion of nestin-immunoreactive cells in a dose-dependent fashion (Figs. 5A–5D and 5F). Half-maximal effects occurred at a dose of 5 nM. By contrast, treatment with Shh-N had no significant effect on the elaboration of GFAP-immunoreactive cells (Figs. 5A, 5B, and 5F). Treatment of Shh-N also increased the total number of cells (Fig. 5E), consistent with prior observations that the factor enhances progenitor cell proliferation (Fig. 4). The increase in neurons and oligodendroglia along with the concomitant decrease in the number of undifferentiated (nestin-immunoreactive) cells suggests that Shh-N enhances both neuronal and oligodendroglial differentiation of neural progenitor cells.

Since it is possible that the generation and propagation of progenitor cells as neurospheres change their response properties, the effects of Shh-N were also examined in primary, unpassaged cultures of embryonic SVZ/striatum. Dissociated E15.5 SVZ/striatal cells were plated on PDL-coated coverslips in the presence or absence of bFGF (10 ng/ml) with and without Shh-N (5 nM). After 6 DIV,

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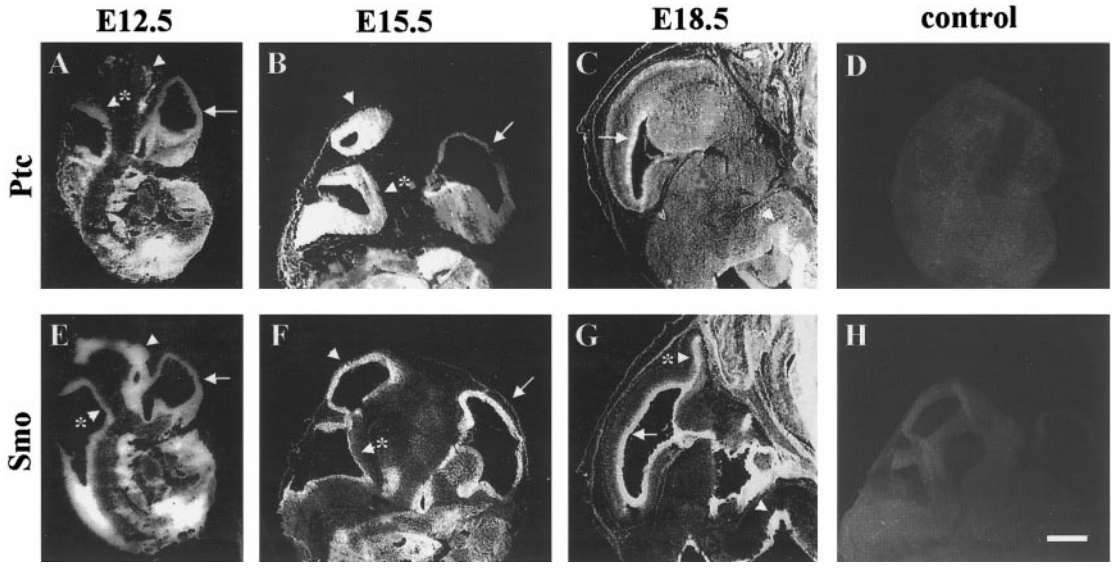

FIG. 2. Expression of Ptc (A, B, C) and Smo (E, F, G) mRNAs as detected by *in situ* hybridization in sagittal sections of E12.5 (A, E), E15.5 (B, F), and E18.5 (C, G) rats. Intense labeling of Ptc mRNA is seen within the forebrain (arrow), midbrain (arrowhead), and hindbrain (arrowhead with asterisk) of E12.5 (A) and E15.5 (B) and also within the SVZ (arrow) and tegmentum (arrowhead) of E18.5 (C). The expression of Smo mRNA is also seen within the forebrain (arrow), midbrain (arrowhead), and hindbrain (arrowhead with asterisk) of E12.5 (E) and E15.5 (F) and within the SVZ (arrow), tegmentum (arrowhead), and olfactory bulb (arrowhead with star) of E18.5 (G). D (E12.5) and H (E15.5) are control sections hybridized with Ptc (D) or Smo (H) sense probe showing background labeling. Scale bar, 1 mm.

immunofluorescence staining was performed using nestin, β-tubulin, O4, and GFAP antibodies. The SVZ/striatal cells were able to survive in the presence of bFGF alone but not with Shh-N alone (Fig. 6A). However, addition of Shh-N along with bFGF further increased cell numbers (Fig. 6A), consistent with findings obtained using neurosphere-derived progenitor cells. Similarly, Shh-N treatment of the primary cultures increased the percentages of both β-tubulin- and O4-immunoreactive cells and reduced the percentage of nestin-immunoreactive cells (Fig. 6B). Furthermore, treatment with Shh-N did not change the proportion of GFAP-immunoreactive cells (Fig. 6B). These observations suggest that Shh-N has the same differentiating effects on primary embryonic SVZ/striatal cells and on progenitor cells passaged as neurospheres.

Cotreatment with Shh-N and BMP2 Inhibits the Astroglial-Inductive, Oligodendroglial-Suppressive, and Anti-proliferative Effects of BMP2

The BMPs, members of a rapidly expanding subclass of the transforming growth factor β (TGF-β) superfamily, participate in a spectrum of cellular inductive events. Our laboratory has demonstrated that BMPs promote the elaboration of astrocytes and inhibit the generation of both oligodendroglial and neuronal lineage species from late embryonic murine SVZ progenitor cells (Gross *et al.*, 1996) and of oligodendroglia from early postnatal rat cortical bipotent oligodendroglial–astroglial (O-2As) progenitor cells (Mabie *et al.*, 1997). Further, the BMPs suppress oligodendroglial differentiation (Mabie *et al.*, 1999) and enhance neuronal differentiation of embryonic VZ progenitor cells (Li *et al.*, 1998, Mabie *et al.*, 1999). In order to determine whether interactions between Shh and BMP signaling modulate progenitor cell differentiation, E12.5 forebrain-derived neurospheres were dissociated and plated on PDL-coated coverslips in the presence or absence of Shh-N (5 nM) and BMP2 (20 ng/ml). bFGF (10 ng/ml) was added initially for 2 days to maintain cell survival. After 6 DIV, immunofluorescence staining was performed using nestin, β-tubulin, O4, and GFAP antibodies. Treatment with BMP2 alone significantly reduced cell numbers, consistent with prior observations that BMP treatment inhibits progenitor cell proliferation (Gross *et al.*, 1996; Zhu *et al.*, 1999), whereas treatment with Shh-N significantly increased cell number (Fig. 7A). However, cell numbers after treatment with both Shh-N and BMP2 did not differ from the control, suggesting that the anti-proliferative effects of BMP2 were negated by the proliferative effects of Shh-N. Application of BMP2 resulted in a dramatic increase in the percentage of astroglial species (GFAP-immunoreactive cells), suppression of oligodendroglial lineage elaboration, and a decrease in the proportion of uncommitted progenitor species (Fig. 7B). Shh-N inhibited both the astroglial-inductive effects and the oligodendroglial-suppressive effects of BMP2 (Fig. 7B). BMP2 and Shh-N each reduced the percentage of nestin immunoreactive cells, suggesting that both factors promote cellular differentiation from these

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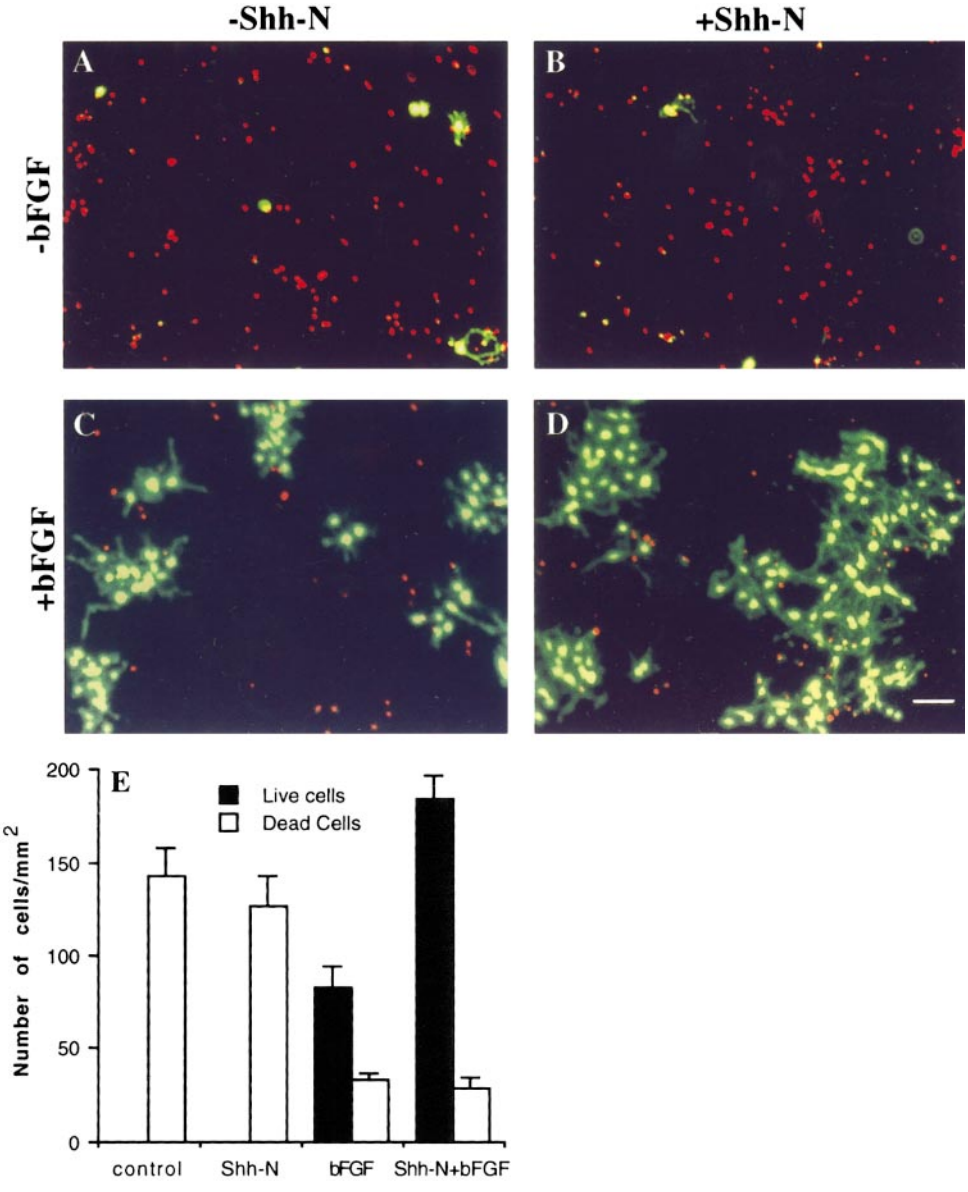

FIG. 3. Shh-N is not a survival factor for neural progenitor cells. Dissociated cells from E12.5 forebrain were cultured in SFM with bFGF (10 ng/ml) + heparin (2 μg/ml) to generate neurospheres. After 6 DIV, bFGF-generated progenitor neurospheres were dissociated with 0.025% trypsin and plated on PDL-coated coverslips in the absence or presence of bFGF (10 ng/ml) and Shh-N (5 nM). Live/Dead staining was performed after 2 DIV. (A–D) Photomicrographs of live (green)/dead (red) staining. Scale bar, 50 μm. (E) The numbers of live/dead cells were counted and are presented as the means ± SEM.

progenitor species. These observations suggest that Shh and BMP2 exert opposing effects on progenitor cell proliferation and differentiation.

DISCUSSION

These observations demonstrate that Shh promotes both the proliferation and the differentiation of embryonic forebrain and diencephalic progenitor cells. Our finding that progenitor cells express the Shh receptor molecules, Ptc and Smo, suggests that these effects may reflect a direct action of Shh. However, it is possible that Shh stimulates production of another factor(s) that is actually responsible for some or all of the proliferative and/or differentiating effects on neural progenitor species. For example, Shh exerts a variety of different cellular effects associated with the anteroposterior polarity of developing chick limb buds that are depen-

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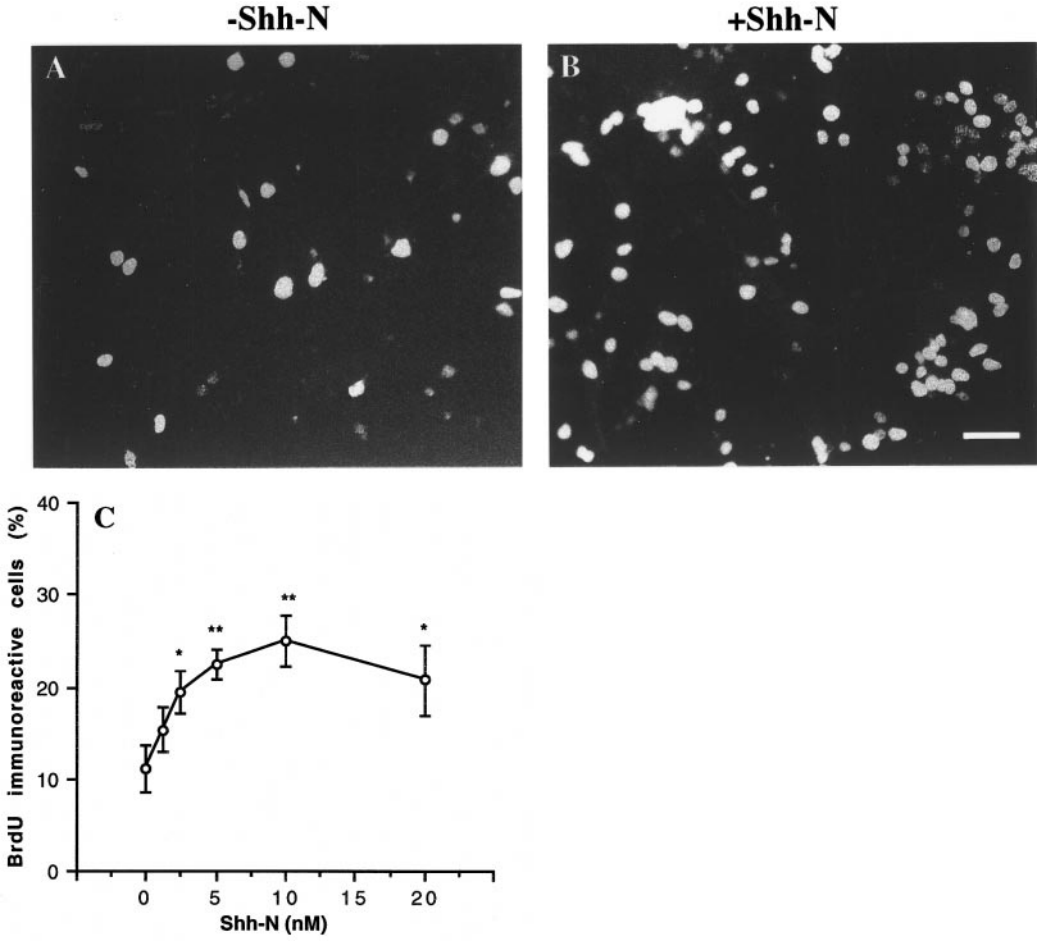

FIG. 4. Shh-N increases the proliferation of neural progenitor cells cultured in the presence of bFGF. bFGF-generated progenitor neurospheres from E12.5 forebrain were dissociated and plated on PDL-coated coverslips in the presence bFGF (10 ng/ml) + different doses of Shh-N. BrdU staining was performed after 2 DIV. Hoechst staining (not shown) was utilized to determine total cell number for calculation of the percentages of BrdU-immunoreactive cells under each experimental condition. (A and B) Photomicrographs of BrdU staining comparing bFGF (10 ng/ml) alone and bFGF (10 ng/ml) + Shh-N (5 nM). Scale bar, 50 μm. (C) The percentages of BrdU-immunoreactive cells are presented as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to −Shh-N.

dent upon the dose, timing, and distance of the source of the factor from the target tissue (Yang *et al.*, 1997). This suggests that at least some of the effects of Shh may be mediated by other signals that are induced by or interact with the factor (Yang *et al.*, 1997). Similarly, the inductive effects of Shh on oligodendroglial lineage commitment in the spinal cord may depend upon additional signals present within the notochord (Orentas and Miller, 1996; Pringle *et al.*, 1996). Cultured neural progenitor cells synthesize and release BMPs, and addition of noggin to such cultures enhances oligodendroglial differentiation (Mabie *et al.*, 1999). Since Shh is known to induce noggin expression (Hirsinger *et al.*, 1997), it is possible that the oligodendroglial-inductive effects that we observed are a result of suppression of BMP signaling rather than a direct effect of Shh.

At least some of the actions of Shh in inducing neural phenotypes are postulated to be direct actions; for example, Shh is involved in induction of floor plate and motor neurons in the hindbrain (Roelink *et al.*, 1995; Tanabe *et al.*, 1995) and basal structures in the telencephalon (Kohtz *et al.*, 1998). However, the inductive effects of Shh are also clearly modulated by signaling from other growth factors. For example, combined signaling of Shh and FGF8 in the mid/hindbrain induces a dopaminergic phenotype in neurons, whereas the confluence of Shh and FGF4 signaling in hindbrain induces a serotonergic cell fate (Ye *et al.*, 1998). Since bFGF was necessary to maintain survival of progenitor cells in our cultures, it is possible that some of the observed effects reflect the combined actions of SHH and bFGF. SHH is produced by midline cells along the entire neuraxis including the telencephalon (Wechsler-Reya and

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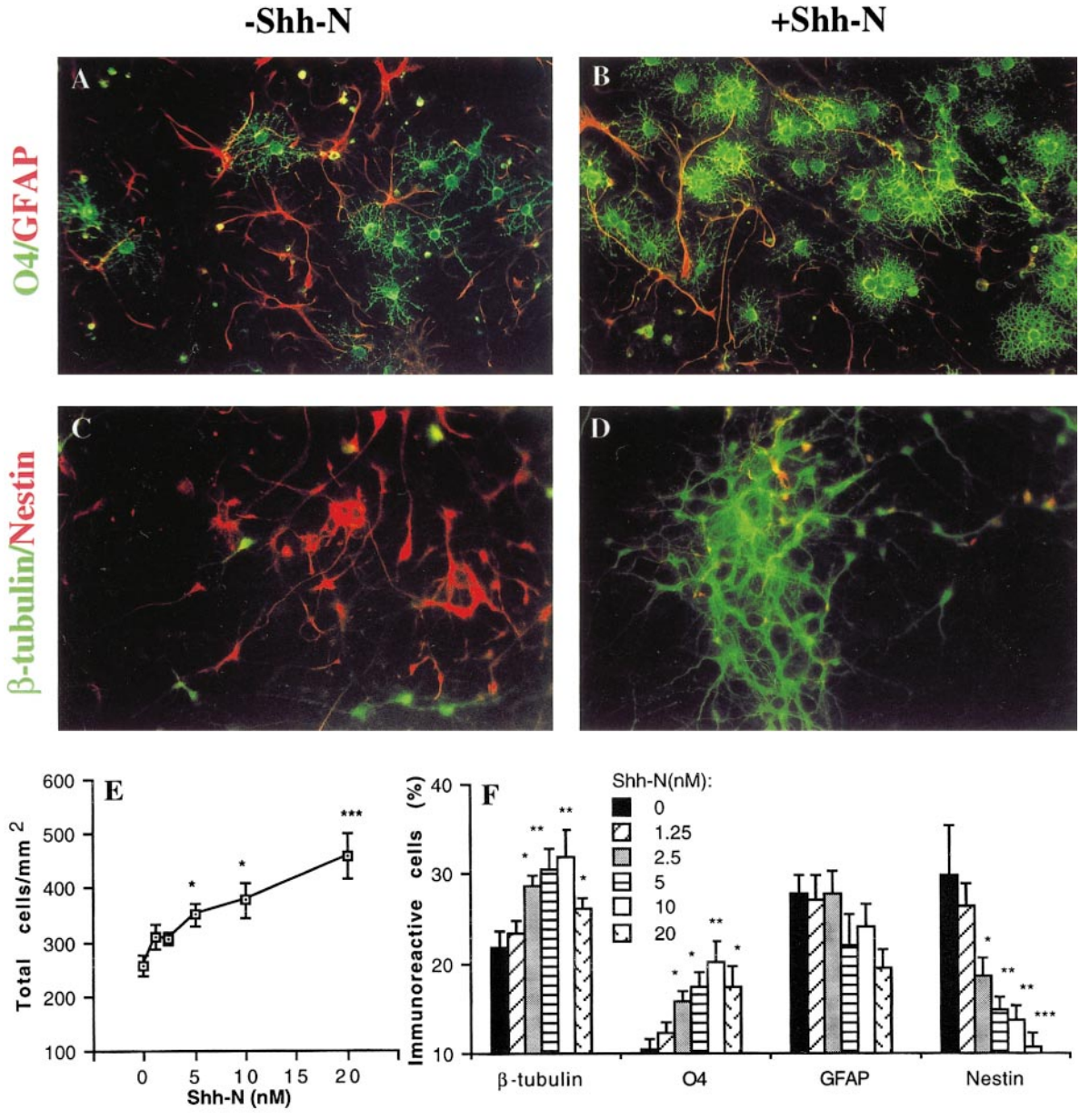

FIG. 5. Shh-N enhances elaboration of both neuronal and oligodendroglial lineage species from bFGF-generated neural progenitor cells. bFGF-generated progenitor neurospheres from E12.5 forebrain were dissociated and plated on PDL-coated coverslips in the presence of bFGF (10 ng/ml) + different doses of Shh-N. After 2 DIV, bFGF was removed and the cells were cultured in the presence of different doses of Shh-N (added every other day) for an additional 6 days. Immunostaining for β-tubulin, nestin, O4, and GFAP was performed. (A–D) Double-immunofluorescence microscopy for GFAP (red) and O4 (green) (A, B), and double-immunofluorescence microscopy for nestin (red) and β-tubulin (green) (C, D). (A and C) −Shh-N. (B and D) +Shh-N (5 nM). Scale bar, 50 μm. (E and F) The number of total cells (E) and the percentages of these immunoreactive cells (F) are presented as the means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to −Shh-N.

Scott, 1999), but is not produced by the multipotent progenitor cells used in this study. This reflects the dissection plane of the ventricular zone that preferentially included more lateral tissues to avoid contamination by extraneous cell types. This suggests that SHH produced by the more midline cells regulates progenitor cell numbers and fate in more lateral regions of the ventricular zone.

Shh is mitogenic for a number of cell types including retinal progenitor cells (Jensen and Wallace, 1997; Levine *et al.*, 1997), differentiated muscle cells (Duprez *et al.*, 1998),

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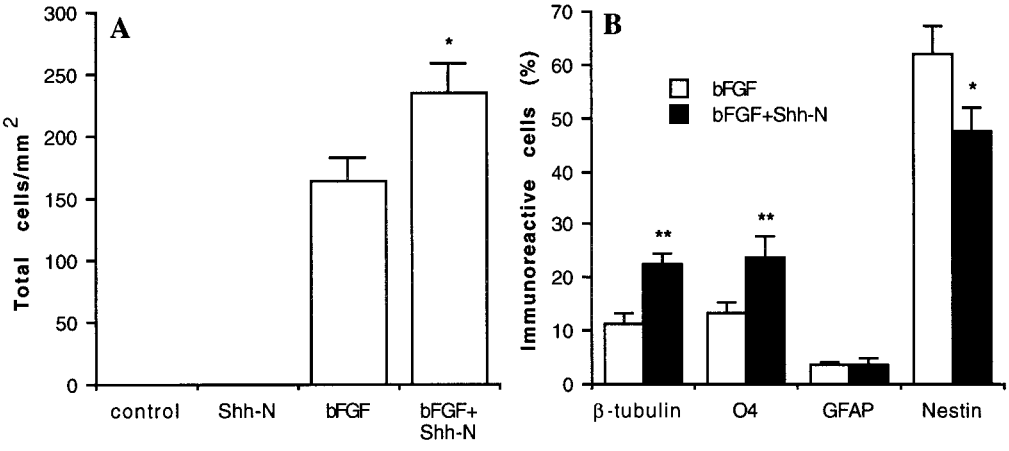

FIG. 6. Shh-N increases elaboration of both neuronal and oligodendroglial lineage species from primary E15.5 striatal cells exposed to bFGF and Shh-N. Dissociated E15.5 striatal cells were plated on PDL-coated coverslips in the presence or absence of bFGF (10 ng/ml) ± Shh-N (5 nM). After 6 DIV, immunostaining for β-tubulin, O4, GFAP, and nestin was performed. The number of total cells (A) and the percentages of the immunoreactive species (B) are presented as the means ± SEM. $*P < 0.05$, $**P < 0.01$ compared to bFGF alone.

lung mesenchymal cells, (Bellusci *et al.*, 1997), and cerebellar granule cell precursors (Wechsler-Reya and Scott, 1999), and our observations indicate that Shh is also a mitogen for embryonic multipotent neural progenitor cells. This raises the possibility that some of the shift in the proportions of differentiated progeny observed after treatment with Shh-N reflected preferential proliferation of specific cellular subpopulations. However, the decrease in uncommitted (nestin-immunoreactive) cells associated with Shh-N treatment (Figs. 5 and 7) strongly supports a role for the factor in promoting cellular differentiation rather than selective proliferation. Further, similar results were obtained using primary cultures and cultures derived from proliferative progenitor neurospheres, suggesting that cellular expansion per se did not alter the pattern of lineage commitment. SHH is unique among the known mitogens for multipotent progenitor cells in that the mitogenic effects are not associated with an effect on survival (Figs. 3 and 6). This suggests that SHH may be involved in expansion of specific progenitor cell pools whose survival is regulated by other regional factors.

Shh-N promoted both neuronal and oligodendroglial differentiation in our cultures. There are several possible explanations for these diverse cellular responses to the

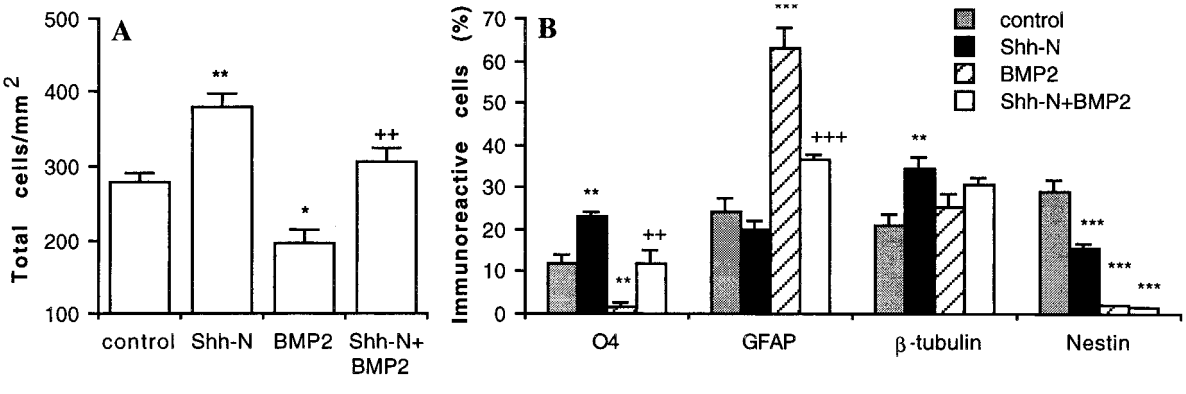

FIG. 7. Cotreatment with Shh-N and BMP2 inhibits the astroglial-inductive and oligodendroglial-suppressive effects of BMP2. bFGF-generated progenitor neurospheres derived from E12.5 forebrain were dissociated and plated on PDL-coated coverslips in the presence of bFGF (10 ng/ml) ± Shh-N (5 nM) ± BMP2 (20 ng/ml). After 2 DIV, bFGF was removed and the cells were cultured in the presence or in the absence of Shh-N (5 nM, added every other day) and BMP2 (20 ng/ml) for an additional 6 days. Immunostaining for β-tubulin, O4, GFAP, and nestin was performed. The number of total cells (A) and the percentages of the immunoreactive species (B) are presented as the means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to control. $++P < 0.01$, $+++P < 0.001$ compared to BMP2.

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factor. First, it is possible that there are different subpopulations of progenitor cells in our cultures with different potentialities and responses to exogenous signals; selective proliferation as discussed above is a corollary of this hypothesis. Although this remains a possibility, several lines of evidence mitigate against it. Prior to plating, more than 99% of the cells in our progenitor cell neurospheres are nestin-immunoreactive in the absence of immunoreactivity using markers of oligodendroglial, neuronal, or astroglial lineage species (Zhu *et al.,* 1999). These observations indicate that the cultured progenitor cells are not yet committed to specific neural lineages. Further, it is possible to shift most of these cells into differing phenotypic fates (Gross *et al.,* 1996; Marmur *et al.,* 1998; Zhu *et al.,* 1999), indicating that they are multipotential rather than committed to specific phenotypes. Finally, there are parallel dose-related increases in both neurons and oligodendroglia in response to Shh-N; if the two phenotypes were derived from different progenitors, different dose–response profiles would likely be anticipated. Alternatively, since it is known that different concentrations of Shh may lead to alternative pathways of differentiation (Roelink *et al.,* 1995), the diverse actions of the factor on neural progenitor cell lineage commitment could reflect dose-related effects. However, this also seems unlikely since increasing doses of Shh-N did not bias cells toward one or the other phenotype, but rather enhanced elaboration of both neurons and oligodendroglia in a dose-dependent manner. Finally, it is possible that some of the effects of Shh-N (e.g., neuronal lineage elaboration) reflect a direct action of the factor, whereas other effects (e.g., oligodendroglial lineage commitment) are due to the induction of other factors such as noggin.

Most of the known effects of Shh during neural development occur relatively early in embryogenesis and involve midline and posterior structures of the brain including the basal structures of the telencephalon (Kohtz *et al.,* 1998). Nevertheless, both the factor (Echelard *et al.,* 1993; Dale *et al.,* 1997; Kohtz *et al.,* 1998) and its receptors (Fig. 2; and Yung *et al.,* unpublished data) are widely expressed in or near paramedian germinal zones throughout the developing brain and are present even in the adult (Miao *et al.,* 1997; Traiffort *et al.,* 1998). The presence of both the factor and its receptor at defined loci during development and the potent effects of Shh on progenitor cell differentiation suggest that the factor may be important for the specification of cellular phenotypes throughout embryogenesis and possibly during the postnatal period.

We find that Shh and BMP2 exert opposing effects on both proliferation and lineage commitment of cultured embryonic progenitor cells. Both inhibitory and synergistic interactions between Shh and the BMPs are important for patterning of the nervous system and for the generation of cellular phenotypes. For example, Shh appears to mediate the ventralizing effects of the notochord, whereas the BMPs exert dorsalizing effects on the neural plate (for reviews see Roelink, 1996; Tanabe and Jessell, 1996). Similarly, in patterning of the medial dermomotome Shh represses *wnt*11 (a marker for the medial compartment of dermomyotome), whereas BMP4 induces formation of the medial lip (Marcelle *et al.,* 1997). However, there are also more complicated interactions in which combined Shh signaling and BMP signaling cooperate to induce intermediate phenotypes, such as in the induction of forebrain ventral midline cells (Dale *et al.,* 1997). The nature of the interactions between BMP and Shh are highly dependent upon other factors. For example, Shh can either induce expression of BMPs (Roberts *et al.,* 1995) or induce expression of the BMP inhibitor, noggin (Hirsinger *et al.,* 1997), depending upon the appropriate cellular context.

It is particularly intriguing in this study that Shh and BMP2 exerted opposing actions on multiple aspects of progenitor cell proliferation and differentiation. Shh was a mitogen, and BMP2 inhibited this effect. Shh promoted oligodendroglial and neuronal lineage commitment without an effect on astroglial development (although Shh inhibited the astroglial-inductive effects of BMP2). Conversely, BMP2 inhibited the oligodendroglial- and neuronal-inductive effects of Shh and promoted astroglial lineage commitment. These observations suggest that the factors may exert mutually opposing effects on a common signaling pathway that regulates multiple aspects of progenitor cell development. In *Drosophila* both negative and positive lineage fate decisions are controlled by the gene *glial cells missing* (*gcm*) (Hosoya *et al.,* 1995; Jones *et al.,* 1995; Akiyama-Oda *et al.,* 1998) and its downstream targets, *tramtrack* and *pointed* (Klaes *et al.,* 1994; Giesen *et al.,* 1997; Hummel *et al.,* 1997). Although mammalian homologues of *gcm* have been described (Altshuller *et al.,* 1996; Kim *et al.,* 1998), they have not been demonstrated to be involved in similar types of switching between choices of cellular phenotype. However, our observations suggest that similar types of mammalian signaling molecules may exist and that Shh and BMP2 may exert opposing regulatory effects on them.

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