SVZa neural stem cells differentiate into distinct lineages in response to BMP4

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Abstract

Neural stem cells (NSCs) reside in the anterior portion of the forebrain subventricular zone (SVZa) and generate the progenitors which will differentiate into neurons, and via a tangential migratory pathway, known as the rostral migratory stream (RMS), migrate to the olfactory bulbs (OB). Bone morphogenetic proteins (BMPs) play significant roles in neural development at different stages and locations, but their roles have not been determined in the SVZa. To explore possible roles of BMPs in SVZa NSCs, BMP4 at various concentrations were tested for their capacity to induce SVZa NSCs. The expression of BMP4 was also examined in living cells using a reportor vector, in which the BMP4 promotor was conjugated with red fluorescent protein (RFP). In the meantime, the differentiation of SVZa NSCs was dynamically monitored by using reportor vectors of the Nestin enhancer and the promoters of TH and GFAP. In the OB, high expression of BMP4 was found using both promoter activity analysis and in situ hybridization. However, low BMP4 expression was found in the RMS and only moderate expression of BMP4 was displayed in the SVZa. The results also demonstrated that low concentrations (1–5 ng/ml) of BMP4 promoted the proliferation of SVZa NSCs but high concentrations (10–100 ng/ml) of BMP4 inhibited this proliferation. BMP4 enhanced neuron commitment before 4 days but inhibited it after 4 days. As the antagonist of BMP4, Noggin almost completely blocked all these BMP4 responses. Thus, our findings indicate that BMP4 promotes the exit from the cell cycle and triggers the differentiation of neuron progenitors in the OB. BMP4 also promotes the proliferation of the committed neuron progenitors in the RMS, but in the SVZa, BMP4 may facilitate the commitment of NSCs into astrocytes.

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Introduction

Much excitement has been generated by the identification of adult brain regions harboring the neural stem cells (NSCs) and their continual generation of new neurons throughout life. One of the most concentrated region of adult NSCs, known as the SVZ, surrounds the postnatal ventricle (review in Alvarez-Buylla and Garcia-Verdugo, 2002; Gould and

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Gross, 2002; Luskin et al., 1997a,b; Marshall et al., 2003). NSCs are located within a discrete region of the anterior part of the neonatal and postnatal subventricular zone, that is, the SVZa, and are the source of an immense number of neurons which are destined to be interneurons of the granule cell and glomerular layers of the OB (Luskin, 1993). These SVZa-derived progenitors migrate to the OB as a network of tangentially oriented chains that converge in a highly restricted pathway called the rostral migratory stream (RMS) (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Luskin, 1993). The periglomerular (PG) and granule cells differentiating from these SVZa-derived progenitors express the neurotransmitter GABA, and about 70% of the
PG cells are dopaminergic neurons which constitute the largest population of dopaminergic neurons in CNS (Betarbet et al., 1996; McLean and Shipley, 1988). The SVZa-derived progenitors have been well characterized and contain an essentially pure population of neuronal progenitors, in contrast to the posterior subventricular zone (SVZp), where mainly astrocytes are produced. Hence, SVZa NSCs are a reliable model for neural development and regeneration (Luskin et al., 1997a; Rakic, 2002).

Bone morphogenetic proteins (BMPs), the largest group of ligands in the transforming growth factor β superfamily, play important roles in vertebrate neurogenesis. Many studies have demonstrated that BMPs and their antagonists including Noggin, Chordin, and Follistatin induce neurogenesis (Garcia-Verdugo et al., 1998; Lim et al., 2000), neurotrophism (Espejo et al., 1999; Hattori et al., 1999; Jordan et al., 1997; Reiriz et al., 1999), neural apoptosis (Graham et al., 1994; Song et al., 1998), and neurospecification both in the peripheral (Fann and Patterson, 1994; Reissmann et al., 1996) and central nervous system (Galter et al., 1999; Lopez-Coviella et al., 2000). BMPs may alternatively stimulate (Li et al., 1998) or inhibit (Li and Reissmann et al., 1996) and central nervous system (Galter et al., 1999; Lopez-Coviella et al., 2000). BMPs may facilitate the commitment of NSCs into astrocytes. Various and sometimes conflicting results have been reported depending on the stage of development (Grinspan et al., 2000; Gross et al., 1996), the distance from the source (Mehler et al., 1997), or the expression of particular transcription factors in target cells (Sun et al., 2001). These different results suggest that modulation of the differentiation of NSCs by BMPs may involve multiple mechanisms. While SVZa-derived cells provide a nice model to explore the proliferation and differentiation of NSCs, the response to BMPs remains not fully understood in the SVZa.

In the study, the expression of BMP4 was detected continuously in living SVZa, RMS, and OB cells by using a reporter vector, in which the BMP4 promoter was conjugated with the red fluorescent protein (RFP). Different Noggin-sensitive effects were observed when SVZa NSCs were exposed to various concentrations of BMP4 at different stages. The differentiation of SVZa NSCs was dynamically observed by using reporter vectors of the Nestin enhancer and the promoters of TH and GFAP. Our findings indicate that BMP4 promotes the exit from the cell cycle and triggers the differentiation of neuron progenitors in the OB. BMP4 also promotes the proliferation of the neuron-committed progenitors in the RMS, but in the SVZa, BMP4 may facilitate the commitment of NSCs into astrocytes.

Materials and methods

In situ hybridization

The intact brains of neonatal mice (P0) were harvested, put into 4% paraformaldehyde in PBS for fixation overnight, and then transferred into a 30% saccharose solution. Sagittal frozen sections (20 µm) were prepared after 48 h and washed twice with PBS. The following steps were performed according to the supplier’s specification (Boster Biological Technology Company, Wuhan, China): the sections were incubated in 0.75% hydrogen peroxide/methanol solution (1:50, 30% H2O2) for 20 min, washed with PBS and immersed into the prehybridization solution at 42°C for 4 h. Biotinylated BMP4 probes were added for hybridization at 42°C overnight. The sections were further washed with 2× standard saline citrate (SSC), 0.5× SSC and 0.2× SSC, respectively, for 15 min each time. After blocking with goat serum, the sections were incubated with biotinylated anti-digoxin antibody at 37°C for 1 h, washed with PBS and then incubated with SABC at 37°C for 30 min. After rinsing with PBS, biotinylated peroxidase was added to the sections followed by an incubation at 37°C for 30 min. Finally, the sections were visualized with 3,3’-diaminobenzidine (DAB), dehydrated through an alcohol series and mounted with neutral balsam for microscopic observation.

Plasmid construction

pDsRed-BMP4pro plasmid construction: BMP4 promoter plasmid was a kind gift from Professor Ebara (School of Medicine, Shinshu University, Japan). A 1.7-kb fragment of the BMP4 promoter was inserted into the pCDNA3.1 vector (the transitional pDNA3.1 vector was constructed due to limitations in the enzyme cutting sites) after digestion with KpnI and XbaI, and then into a promotorless pDsRed2-1 vector (Clontech) after digestion with HindIII and ApaI. The successful ligation was confirmed by double cutting.

pEGFP-Noggin construction: after the Noggin plasmid (from Professor Cai) was digested with EcoRI and ApaI, a 1.3-kbp Noggin fragment was inserted into the pEGFP-C2 vector (Clontech) and double cutting was used to verify the successful ligation. Nestin-GFP plasmid (Constructions of Nestin enhancer and GFP) and TH-GFP plasmid (Constructions of TH promoter and GFP) were offered by Japan Science and Technology Corporation. GFAP-GFP (Constructions of GFAP promotor and GFP) was a kind gift from Professor Morita.

Cell culture

The procedures to obtain SVZa NSCs have been previously described (Law et al., 1999; Luskin et al., 1997a,b; Zigova et al., 1996). Briefly, P0 mouse pups were fixed in wax plates and the epicraniums, skulls, and meninges were removed and the whole brain was isolated. Under the guidance of a dissecting microscope, the sagittal and longitudinal slices (about 1 mm) along the margins of the OB were obtained. The SVZa (adjacent to the anterior horn of lateral ventricle, more transparent appearance compared with the overlying corpus callosum) was identi-
fied under the microscope. Single cell suspension was prepared using mechanical dissociation with the blow of Pasteur pipettes. After counting living cells with trypan blue, 1 \times 10^6 cells were seeded into culture flasks (75 ml) and cultured in serum-free medium (DMEM/F12 1:1) supplemented with B27 (Gibco) and bFGF (20 ng/ml, Sigma). After the formation of primary clones, single cell suspension was prepared by mechanical dissociation of these primary clones and 1 \times 10^6 cells were again seeded into culture flasks. The cell passaging was performed by mechanical dissociation every 5–7 days according to the above-mentioned methods.

SVZa NSCs that passaged over three times were harvested and the cell number of each sample adjusted to 4 \times 10^6. After centrifugation at 800 rpm for 5 min, the supernatant was removed. Then, NSCs were transfected with the pDsRed-BMP4pro plasmid using Nucleofector™ (Mouse NSC Nucleofector™ Kit was purchased from Amaxa) through the following steps: 100 \mu l mouse NSC Nucleofector Solution and 5 \mu g DNA were added into an Amaza certified cuvette for transfection using Nucleofector™ with the selection of A-33 program. The cells were seeded in 6-well plates (1 \times 10^6 cells each well) and cultured in the presence of 10% fetal bovine serum (Hyclone). Then, the cells were dynamically observed under the fluorescence microscope and some cells fixed for immunofluorescence staining of MAP-2 and GFAP. Cells in the SVZa, RMS and OB were isolated under the dissecting microscope, cultured for 3 days in the NSCs medium and then transfected with pDsRed-BMP4pro plasmid using Nucleofector™. The cells were cultured in the DMEM/F12 medium supplemented with 10% fetal bovine serum before again observation under the fluorescence microscope. The spectrofluorometry was performed at 48 h.

SVZa NSCs that passaged over three times were incubated with either 1, 2, 5, 10, 20, 50, or 100 ng/ml BMP4, cultured for 7 days, digested with 0.25% panreatin, and counted. At day 3, some cells that were harvested for flow cytometry (FCM) measurement were washed in PBS, fixed in 75% ethanol for 48 h and stained with propidium iodoide (PI). SVZa NSCs were seeded in 6-well plates and supplemented with 1, 2, 5, 10, 20, 50, or 100 ng/ml BMP4 and 10% fetal bovine serum (DMEM/F12 1:1). At days 2 and 7, MAP-2 and NSE immunofluorescence staining was performed. NSE fluorescent-positive cells were detected by FCM. Meanwhile, SVZa NSCs were seeded into 6-well plates, incubated with 10 ng/ml BMP4, and cultured in DMEM/F12 medium supplemented with the 10% fetal bovine serum. At days 1, 2, 3, 4, 5, 6, 7, or 8 after culture, NSE immunofluorescence staining and FCM were performed. SVZa NSCs were transfected with pE GFP-Noggin according to the Nucleofector™ protocol as described above and incubated with 1, 2, 5, 10, 20, 50, or 100 ng/ml BMP4 as well as 10% fetal bovine serum for culture. Thereafter, NSE immunofluorescence staining was performed at days 2 and 7. Some cells were harvested for FCM detection.

SVZa NSCs were harvested and transfected with Nestin-GFP, GFAP-GFP and TH-GFP based on the Nucleofector™ protocol. The transfected NSCs were then cultured in 10% fetal bovine serum supplemented with (or not) 10 ng/ml BMP4. The expression of GFP was observed dynamically and detected with the spectrofluorometer at days 1, 2, 3, 4, 5, 6, or 7. The concentration of GFP was calculated according to the standard curve.

**Immunofluorescence staining**

Coverslips containing cultured cells were fixed in 4% paraformaldehyde in PBS for 15 min, then incubated in TritonX-100 (0.4%) and BSA (0.1%) at 37°C for 15 min before washed with 0.1 M PBS. The primary antibody against MAP-2 (Chemicon, 1:200), GFAP (Chemicon, 1:200) and NSE (Promega, 1:200) was added, and the coverslips were kept in the moist chamber at 4°C for 12 h. Primary antibody binding was detected following incubation with fluorescently labeled secondary antibody (TRITC and FITC, from Promega, 1:200) at 37°C for 1 h. The coverslips were mounted with 50% glycerol and immediately observed under the fluorescence microscope (Olympus). To demonstrate specificity, the primary or secondary antibodies were omitted in the separate control coverslips.

**FCM detection**

Cells were digested with 0.25% pancreatin, rinsed with D-Hank’s solution and centrifuged at 1500 rpm for 5 min, which was repeated three times. NSE immunofluorescence staining was then performed. The samples were fully mixed and 3 samples in each group used for the FCM detection (type PLUS of FACstar, Becton Dickinson, USA).

**Spectrofluorometry**

Cells were digested with 0.25% pancreatin and harvested (1 \times 10^6 cells in each sample). Each sample was added into a 4-ml sonication buffer solution and frozen in liquid nitrogen, later thawed on warm water and placed on ice for 1 min, which was repeated 5 times. After high-speed centrifugation at 4°C, the supernatant was analyzed using spectrofluorometer (GFP: excitation at 488 nm and emission at 510 nm; RFP: excitation at 561 nm and emission at 587 nm).

**Statistical analysis**

Chi-square tests and independent-samples t-tests were performed using the SPSS software pack.
Results

Expression of BMP4 mRNA in neonatal mouse brain

BMP4 mRNA was extensively distributed in the neonatal mouse brain, especially the cerebral cortex, midbrain and cerebellum (Fig. 1A). Along the migratory pathway from the SVZa to the OB, BMP4 mRNA was highly expressed in the peripheral cells of the OB, especially in the mitral cell layer and, to a lesser extent, in the periglomerular and granular cell layers. However, in the central area of the OB, where the neuron progenitors reached, the expression of BMP4 mRNA was low (Figs. 1B and C). BMP4 mRNA expressed moderately with sporadic distribution in the cells of the SVZa, but high in the astrocyte sheath enveloping SVZa (Fig. 1E). In the RMS, the expression of BMP4 mRNA was relatively low, and only a few positive cells could be observed (Fig. 1D). In addition, the expression of BMP4 mRNA in the SVZp region, adjacent to the SVZa, was rather high (Fig. 1F).

Analysis of BMP4 promotor activity

BMP4 promotor was conjugated with RFP to visualize the promotor activity and provide an indirect measurement of the expression of BMP4. After the pDsRed-BMP4pro plasmid was introduced into SVZa NSCs, fetal bovine serum was immediately added to induce differentiation. No positive cells were observed 4 h later but a small number of red fluorescence cells with strong fluorescent intensity and distinguishable cell processes were seen at 12 h (Fig. 2A). After 24–48 h, the number of positive cells apparently increased, and the expression of fluorescent protein reached a maximum at 48 h (Figs. 2B and C), after which fluorescence decreased and was almost absent after 5 days. RFP was mainly expressed in neurons within 48 h (Figs. 2E and 3A) with thick and long processes, but in astrocytes (Figs. 2F and 3A) at later periods.

To more closely imitate the in vivo situation, the cells from SVZa, RMS, and OB were cultured for only 3 days, when the majority of cells were progenitors (Brazel et al., 2003; Doetsch et al., 1997). RFP expression during the differentiation of progenitors derived from SVZa, RMS, and OB was similar to that of SVZa NSCs. Similar to results seen by in situ hybridization, there also existed significant regional differences in BMP4 promotor activity. There were clearly more positive cells in the OB than in the SVZa and fluorescence appeared mainly in the neuron-like cells (Fig. 2H). There were relatively less fluorescence cells in the RMS (Fig. 2G). The results of spectrofluorometry showed that the fluorescence intensities in the OB, SVZa, and RMS were 39.72 ± 2.65, 33.93 ± 2.99, and 23.16 ± 0.14, respectively (Fig. 3B). These values were significantly different (P < 0.05).

Fig. 1. The expression of BMP4 mRNA in the neonatal (P0) mouse brain shown by in situ hybridization. (A) In sagittal section of the brain, BMP4 mRNA was extensively distributed throughout the neonatal mouse brain, especially high in the cerebral cortex, midbrain and OB. (B–E) In the migratory pathway from SVZa to the RMS and the OB, BMP4 mRNA was highly expressed in OB(C), and next in SVZa (E) and RMS(D). BMP4 mRNA was highly expressed in the peripheral cells of the OB, especially in the mitral cell layer, and to a lesser extent in the periglomerular cell layer and the granular cell layer. BMP4 mRNA was rather low in the central area of the OB (C). (F) In the SVZp, the BMP4 mRNA expression was fairly high. Scale bar = 200 μm.
SVZa NSCs were purified and the effects of BMP4 on cell proliferation was investigated. Cell counting showed that the proliferation of SVZa NSCs was stimulated by BMP4 at concentrations of 1, 2, or 5 ng/ml, with maximal BMP4 effects at 2 ng/ml. However, BMP4 at higher concentrations (10, 20, 50, or 100 ng/ml) inhibited the proliferation of SVZa NSCs (Fig. 4). Cell-cycle analysis with FCM showed decreased cells at phase G1/G0 in response to BMP4 at 1, 2, or 5 ng/ml, but increased number of cells at phase S and an increased G2+M/G0 ratio. BMP4 at 2 ng/ml again produced the maximum effect, with the percentage of cells in G1/G0 phase decreasing from 41.6% (control) to 24.2% and those in S phase increasing to 57.7% (Figs. 5A and B). BMP4 at concentrations of 10, 20, 50, or 100 ng/ml, caused a marked increase in the number of cells at phase G1/G0, while decreasing sharply those at phases M.

Proliferation of SVZa NSCs in response to BMP4

SVZa NSCs were purified and the effects of BMP4 on cell proliferation was investigated. Cell counting showed that the proliferation of SVZa NSCs was stimulated by BMP4 at concentrations of 1, 2, or 5 ng/ml, with maximal BMP4 effects at 2 ng/ml. However, BMP4 at higher concentrations (10, 20, 50, or 100 ng/ml) inhibited the proliferation of SVZa NSCs (Fig. 4). Cell-cycle analysis with FCM showed decreased cells at phase G1/G0 in response to BMP4 at 1, 2, or 5 ng/ml, but increased number of cells at phase S and an increased G2+M/G0 ratio. BMP4 at 2 ng/ml again produced the maximum effect, with the percentage of cells in G1/G0 phase decreasing from 41.6% (control) to 24.2% and those in S phase increasing to 57.7% (Figs. 5A and B). BMP4 at concentrations of 10, 20, 50, or 100 ng/ml, caused a marked increase in the number of cells at phase G1/G0, while decreasing sharply those at phases M.
and S. 79.4% of cells were at G1/G0 phase in response to 100 ng/ml BMP4 while S phase cells were reduced to 2.0% (Fig. 5D). These results indicated that the effects of BMP4 were concentration-dependent and BMP4 could produce opposite responses. For instance, BMP4 at low concentrations could accelerate NSCs entering the cell cycle for continuous proliferation but BMP4 at high concentrations could cause exit from the cell cycle.

The neuron commitment of SVZa NSCs exposed to BMP4

A further study was carried out to investigate the role of BMP4 in differentiation of SVZa NSCs. MAP-2 and NSE immunofluorescence stainings showed that the number of positive cells increased 2 days after the addition of BMP4 at concentrations of 1, 2, 5, 10, 20, 50, or 100 ng/ml (Fig. 6). The FCM measurements revealed that the increase in neurons in response to BMP4 at concentrations of 1–10 ng/ml was gradual and reached a plateau level when the BMP4 was at concentrations of 10–100 ng/ml on day 2. But neuron number reduced on day 7 (Fig. 7A), which was confirmed by MAP-2 and NSE immunofluorescence staining as well as FCM (Figs. 6 and 7A). The results on days 2 and 7 were incompatible, and the previous results demonstrated that 10 ng/ml BMP4 was the saturation concentration; therefore, we added BMP4 at 10 ng/ml to observe the differentiation of SVZa NSCs at different time points. The results of FCM analysis showed that BMP4 increased the number of neurons in the early stage, and this reached a peak at day 2 (at day 4 in the control), thereafter reduced (Fig. 7B).

The antagonism of Noggin

As antagonists of BMPs, Noggin and Chordin play distinct role in patterning the early mouse embryo and Noggin shows the stronger antagonism (Gratsch and O’Shea, 2002). To confirm the specificity of the above response to BMP4, the Noggin-GFP vector was constructed and transfected into SVZa NSCs cultured in media containing various concentrations of BMP4. NSE immunofluorescence staining was then performed. The results showed that Noggin could block the effects of different concentrations of BMP4. The number of neurons in the early stage was reduced, while the number of neurons was increased in the late stage (Fig. 8). This was also validated by the FCM analysis (Figs. 7C and D).

The activities of the Nestin enhancer and the promoters of TH and GFAP in response to BMP4

The above results showed that BMP4 played complex roles in differentiation of SVZa NSCs: BMP4 promoted neuron commitment in the early stage but inhibited it in the late stage. To help provide confirmatory results, we used the Nestin enhancer and the promoters of TH and GFAP to dynamically display the process of differentiation of SVZa NSCs. After transfection with Nestin-GFP, many fluorescent cells appeared at 8 h, in general, without obvious processes although a few positive cells with obvious processes. The number of positive cells reached a maximum at 24 h and thereafter declined. With the addition of BMP4 (10 ng/ml), the number of fluorescence cells decreased markedly. There was a significant difference in the number of fluorescent cells in the presence of 10 ng/ml BMP4 compared with the control group, especially at 24–28 h (Figs. 9A and D). The spectrofluorometry that was performed at day 1, 2, 3, 4, 5, 6,
or 7 showed that, with the addition of BMP4, Nestin enhancer activity was obviously reduced (Fig. 10A).

After transfection of SVZa NSCs with TH-GFP, only a few positive cells emerged at 8 h. Later, the number of positive cells with obvious processes gradually increased and were distributed diffusely. The maximum number of positive cells appeared at 48–72 h, but the total number of positive cells was still low. After BMP4 (10 ng/ml) was added, the number of positive cells increased and after 24–48 h incubation with BMP4, there was a significant increase compared with the control group (Figs. 9B and E). The spectrofluorometry showed a significant difference between BMP4 group and the control group at days 1–3 (P < 0.05), but this difference was insignificant after 4 days (Fig. 10B, P > 0.05). Spectrofluorometry results showed a continuous enhancement of GFAP promotor activity, even after 4 days, when the Nestin enhancer and TH promotor activity remained unchanged with addition of BMP4 at 10 ng/ml (Fig. 10C).

Discussion

Labeling of living cells by fluorescent proteins is now a common scientific technique, with red, blue, and yellow fluorescent proteins utilized in addition to the original green fluorescence protein. The fusion protein technique, by which target genes are inserted into a fluorescent protein vector, can continuously, although indirectly, measure gene expression in the living cells. However, this technique also has some drawbacks; for example, excessive external proteins may influence the physiological activity of the living cells and result in deviations from their normal function (Horn et al., 2002; Spergel et al., 2001). Unlike the immunohistochemistry, the fusion protein technique cannot display the inherent expression of a specific protein. In this study, conjugating the BMP4 promotor with red fluorescent protein reveals the activity of the BMP4 promotor, and cannot only indicate the internal protein expression to some extent but also allow one to continuously trace expression in the living cells. The second intronic enhancer of the Nestin directs its transcription to NSCs, and GFP under the control of the Nestin enhancer can be used to identify NSCs (Keyoung et al., 2001). Similarly, a 9-kb 5′ flanking sequence of the promotor of tyrosine hydroxylase, a pivotal enzyme in the synthesis of dopamine, has been used to recognize the dopaminergic neurons because SVZa NSCs differentiate into dopaminergic neurons after they migrate to the OB (Sawamoto et al., 2001). GFAP is a marker used frequently to identify...
astrocytes, and the specificity of the GFAP promotor to identify astrocytes has been verified (Miura et al., 1990; Tsuchiya et al., 2002). Therefore, these conjugated promoters offer a good method to observe the differentiation of SVZa NSCs.

From neurogenesis to the maturation of the vertebrate nervous systems, BMPs mediate a diverse array of developmental processes including cellular survival, proliferation, morphogenesis, lineage commitment, inhibition of alternate lineages, differentiation, and apoptosis (Mehler et al., 1997; Mabie et al., 1999). BMPs and their receptors are expressed in the adult rat SVZa and the mouse OB (Charytoniuk et al., 2000; Lim et al., 2000; Ming et al., 2002), but their expression has not been reported in the neonatal SVZa, RMS, and OB. The expression of BMPs in the neonate may be more significant because SVZa is highly proliferative in the first two postnatal weeks (Altman, 1969; Bayer, 1983; Brazel et al., 2003; Law et al., 1999). In our study, the in situ hybridization results and the analysis of BMP4 promotor activity demonstrate expression of BMP4, not only in the SVZa and the OB, but also in the RMS. Such patterns of BMP expression have not been previously reported and suggest that BMPs play important roles in the migration of SVZa-derived progenitors in neonatal mice. The results of the hybridization and the promotor activity experiments show that BMP4 expresses at low level in the RMS and that BMP4 at low concentrations promotes proliferation of SVZa NSCs. Hence, in this area, BMP4

Fig. 6. MAP-2 immunofluorescence staining showed the differentiation of SVZa NSCs being exposed to BMP4 at various concentrations. (A–D) At day 2, the neuron commitment was enhanced with the addition of BMP4 at concentrations of 5 ng/ml (B), 10 ng/ml (G), or 100 ng/ml (D) compared with the control group (A, 0 ng/ml). (E–H) At day 7, the extent of neuron commitment was reduced when BMP4 was at concentrations of 5 ng/ml (F), 10 ng/ml (G) or 100 ng/ml (H), in contrast to control group (E, 0 ng/ml). Scale bar = 20 μm.
promotes the proliferation of progenitors rather than undergoes differentiation.

BMPs can promote or inhibit the proliferation of NSCs depending on their concentration. In this study, BMP4 at low concentrations (1–5 ng/ml) promotes the proliferation of NSCs but BMP4 at high concentrations (10–100 ng/ml) inhibits the proliferation (Lillien and Raphael, 2000; Panchision et al., 2001; Shou et al., 2000). BMPs may alternatively stimulate (Li et al., 1998) or inhibit (Li and LoTurco, 2000; Lim et al., 2000; Mabie et al., 1999) brain neuronal differentiation. In spinal cord and neural crest stem cells, BMPs induce the differentiation of motor neurons (Kalyani et al., 1998) and sympathetic neurons (Bronner-Fraser, 2002; Howard et al., 2000).

Fig. 7. Differentiation of SVZa NSCs induced by BMP4 and Noggin was shown by the FCM. (A) BMP4 at various concentrations promoted neuron differentiation at day 2 but inhibited it at day 7. (B) BMP4 at 10 ng/ml increased the number of neurons at the early stage and reached a peak at day 2 (at day 4 in the control) and thereafter reduced. Noggin blocked the actions of BMP4 at different concentrations, showing neuron inhibition at day 2 (C) and neuron promotion at day 7 (D).

BMP4 is a specific effect but not a secondary effect due to BMP4-induced cell death.

The reason that BMP4 has incompatible effects on the differentiation of SVZa NSCs may be because the SVZa-derived cells isolated and cultured in serum-free medium contain NSCs as well as neuron progenitors. At the early stage, BMP4 promotes the committed neuron progenitors to exit the cell cycle and accelerates them to differentiate into neurons, which is confirmed by the following evidences: (1) The results of in situ hybridization show that BMP4 is expressed highly in the peripheral cells of the OB, but is rather lowly in the central area of the OB and the RMS; hence, BMP4 is just expressed in the areas where neuron progenitors exit the cell cycle. (2) The FCM analysis indicates that BMP4 at high concentrations (10–100 ng/ml) promotes SVZa NSCs to exit the cell cycle. (3) FCM detection of NSE immunofluorescence shows that BMP4 promotes neuron differentiation only at early period. (4) Activities of the Nestin enhancer and the TH promotor show that BMP4 decreases the expression of NSCs markers and increases the expression of dopaminergic neuron marker in the early stage, which shows no significant differences in the late stage, indicating that BMP4 accelerates the differentiation of SVZa-derived precursors. The analysis of GFAP promotor activity reveals that BMP4 continuously enhances GFAP promotor activity, which is different for TH promotor. In addition, FCM detection indicates that BMP4 inhibits neuron differentiation at day 7, and the BMP4 promotor activity reveals that RFP is mainly expressed in the astrocytes at the late stage of differentiation. Therefore, the specific action of BMP4 on SVZa NSCs may inhibit
neuron differentiation and promote astrocyte commitment although it may retain neuron commitment in the RMS, which is validated by in situ hybridization showing that abundant BMP mRNA exists in the SVZp, where NSCs are mainly differentiated into astrocytes (Gross et al., 1996). That BMPs promote the exit of precursors from the cell cycle partly accounts for the different results reported in some papers due to different proportions and properties of the committed progenitors isolated in different experiments.

In the OB, high expression of BMP4 is found by the promotor activity analysis and in situ hybridization. BMP4 at high concentrations inhibits the proliferation of SVZa NSCs and promotes their exits from the cell cycle in vitro. Thus, BMP4 forces the committed progenitors out of the cell cycle and initiates differentiation in the OB. That abundant BMP mRNA exists in the SVZp suggests that BMP4 promotes differentiation of NSCs into astrocytes in this region. These complex effects of BMP4 relate to the diversity of BMPs receptors (Helm et al., 2000; Panchision et al., 2001; Stull et al., 2001; Zwijsen et al., 2003), BMP signaling pathways (Derynck, 1998; Ghosh-Choudhury et al., 2002; Hay et al., 2001; Heldin et al., 1997; Mehler et al., 1998; Rajan et al., 2003; Van-Grunsven et al., 2002; Zwijsen et al., 2003), and BMP antagonists (Charytoniuk et al., 2000; Groppe et al., 2002; Smith and Harland, 1992). Noggin almost completely blocks BMP4 action in this

Fig. 8. Differentiation of neurons displayed by the NSE immunofluorescence staining after pEGFP-Noggin was transfected into SVZa NSCs supplemented with various concentrations of BMP4. (The red indicates NSE and the green indicates GFP). (A–D) At day 2, there were less neurons after Noggin was transfected when BMP4 was at concentrations of 5 ng/ml (B), 10 ng/ml (C), or 100 ng/ml (D) compared with the control group (A, 0 ng/ml). (E–H) At day 7, Noggin enhanced neuron commitment when BMP was at concentrations of 5 ng/ml (F), 10 ng/ml (G), or 100 ng/ml (H) compared with the control group (E, 0 ng/ml). Scale bar = 20 μm.
Fig. 9. Change in the activities of the Nestin enhancer and the promotors of TH and GFAP after 10 ng/ml BMP4 was added to induce SVZa NSCs. (A–C) The control group. (D–F) BMP4 decreased Nestin enhancer activity (D vs. A) and reinforced activities of TH promotor (E vs. B) and GFAP promotor (F vs. C) at 48 h. Scale bar = 20 μm.

Fig. 10. Activities of the Nestin enhancer and the promotors of TH and GFAP were detected by the spectrofluorometer. BMP4 decreased Nestin enhancer activity (A) and reinforced TH promotor activity (B) at the early stage (1–3 days, \( P < 0.05 \)), but enhanced the GFAP promotor activity continuously (C, 1–7 days, \( P < 0.05 \)), even after 4 days, when BMP4 had no effect on Nestin enhancer and TH promotor activity (\( P > 0.05 \)). The concentration of GFP was calculated based on the GFP standard curve. Unit: ng/ml.
study, and Lim et al. (2000) have reported that Noggin is expressed in SVZ ependymal cells. Therefore, a balance between BMPs and their antagonists participates in the development of SVZa NSCs. Recently, a BMP-based induction-termination mechanism has been proposed to describe the shift from proliferation to differentiation: BMPs cause proliferation through BMPRIA, and BMPRIA signaling simultaneously induces expression of BMPRIB, and activation of BMPRIB drives mitotic arrest via the CDK inhibitor p21WAF1 and results in differentiation (Panchision and McKay, 2002). The results presented here seem to support a hypothesis that BMP4 at low concentrations promotes proliferation of SVZa NSCs through BMPRIA, but BMP4 at high concentrations triggers the differentiation by BMPRIB.

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