Expression of BMP-2 and BMP-4 proteins by type-1 and type-2 astrocytes induced from neural stem cells under different differentiation conditions

Jian-Guo Hu[1, 2], Yu-Xin Zhang[2], Qi Qi[2], Rui Wang[2], Lin Shen[2], Chen Zhang[2], Jin Xi[2], Jian-Sheng Zhou[2], and He-Zuo Lü[1, 2] *

[1] Central Laboratory, the First Affiliated Hospital of Bengbu Medical College, [2] Anhui Key Laboratory of Tissue Transplantation, Bengbu Medical College, Anhui 233004, P.R. China, *Email: jghu.hzlv@yahoo.com

Bone morphogenetic proteins (BMPs), a subgroup of the TGF-β superfamily, play critical roles in neural progenitor cell fate determination. Neural stem cells (NSCs) are multipotent progenitor cells that can differentiate into neurons, oligodendrocytes and astrocytes under certain conditions. In our recent report, using an antibody that can recognize both BMP-2 and BMP-4 (BMP-2/4), we showed that BMP-2/4 is only expressed in astrocytes differentiated from NSCs in a medium containing 1% fetal bovine serum (FBS). In this *in vitro* model, the astrocytic differentiation of NSCs was mainly toward type-2. When NSCs were cultured in a medium containing 10% FBS, most of the cells differentiated into type-1 astrocytes. However, little information is available for BMP-2 and BMP-4 expression in type-1 and type-2 astrocytes induced from NSCs under these different culture conditions. In this study, using two antibodies specific for BMP-2 and BMP-4, respectively, we discriminated the presence of BMP-2 and BMP-4 in NSCs and their derivatives under 1% and 10% FBS culture conditions by RT-PCR, western blot and immunofluorescence staining. We found that BMP-2 and BMP-4 are highly expressed in both type-1 and type-2 astrocytes, and no detectable expression in NSCs, neurons and oligodendrocytes. This suggests that the astrocytes might be one source of BMPs during the differentiation of NSCs. However, in our model, we cannot exclude the possibility that microglia or endothelial cells could also be a source of BMPs.

Key words: Neural stem cells, bone morphogenetic proteins, differentiation, astrocytes, fetal bovine serum

Neural stem cells (NSCs) are multipotent progenitor cells which possess the ability to self-renew and generate different neural cell types within discrete spatiotemporal developmental windows when they encounter different signals (Abematsu et al. 2006). These precursor cells can be isolated and expanded *in vitro* in the presence of mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Fu et al. 2005, Lu et al. 2008a). Upon removal of the mitogens and provision of appropriate substrates these cells can differentiate into neurons, oligodendrocytes and astrocytes (Hu et al. 2004, Fu et al. 2005, Lu and Hu 2009). In the differentiated cells of NSCs, astrocytes may have two morphologically and antigenically distinct cell types (type-1 and type-2 astrocytes), which were observed originally in primary cultures of astrocytes obtained from the optic nerve (Raff et al. 1983, Raff 1989). Type-1 astrocytes are large and fibroblastlike cells that possess few processes. Type-2 astrocytes are stellate-like cells that have a central body with numerous long processes. Both type-1 and type-2 astrocytes are GFAP-positive, but type-2 astrocytes can be identified by A2B5, a monoclonal antibody against an extracellular ganglioside antigen (Raff et al. 1983, Raff 1989).

In vitro, fetal bovine serum (FBS) is a substance commonly used to induce NSC differentiation. If NSCs are exposed to 1% FBS, they would differentiate into type-2 astrocytes, however, when cultured in a medium supplemented with 10% FBS, they give rise to type-1 astrocytes. In our recent report, using an antibody that can recognize both BMP-2 and BMP-4 (BMP-2/4), we showed that BMP-2/4 is only expressed

Table I

Primer characteristics and PCR parameters

Gene (GenBank no.)	Primer Sequence (5'-3')	Amplicon	Annealing temp.	PCR cycles
BMP-2 (NM_017178)	F: CCAGGTTAGTGACTCAGAACAC R: TCATCTTGGTGCAAAGACCTGC	181bp	51ºC	30
BMP-4 (Z22607)	F: TCTGGTCTCCGTCCCTAATG R: CTGAATCTCGGCGACTTTTT	395bp	51ºC	30
β-actin (NM_031144)	F: ATTGTAACCAACTGGGACG R: TTGCCGATAGTGATGACCT	533bp	55ºC	30

(F) forward; (R) reverse

in type-2 astrocytes under an *in vitro* model in which NSCs were differentiated in a medium containing 1% FBS (Lu and Hu 2009). However, little information is available for BMP-2 and BMP-4 expression in type-1 and type-2 astrocytes induced from NSCs.

In this study, based on different *in vitro* models in which NSCs can differentiate into type-1 and type-2 astrocytes, we discriminated the presence of BMP-2 and BMP-4 in NSCs and their derivatives under different culture conditions by reverse transcription–polymerase chain reaction (RT-PCR), western blot and immunofluorescence staining.

Spinal cord-derived NSCs were prepared as described previously (Fu et al. 2005, Lu and Hu 2009). Briefly, embryonic spinal cords were collected from E14.5 Sprague–Dawley (SD) rats. The cells were isolated by mechanical pipetting in Leibovitz's L-15 medium (Gibco, Grand Island, N.Y.). The suspension was filtered through a nylon mesh of 70 μm. After washing, cells were seeded at a density of 1×10^5 cells/mL, and incubated at 37°C in a humidified 5% CO_2–95% air atmosphere. The culture medium, referred to as basal-NSC-medium, was composed of DMEM/F12 (Gibco), 1% N2 (Gibco), 1% B27 (Gibco), 3 μg/mL heparin (Sigma, St. Louis, MO), and 2 mM glutamine (Gibco), supplemented with 20 ng/mL basic fibroblast growth factor (bFGF, Gibco) and 20 ng/mL epidermal growth factor (EGF, Sigma). At day 3 or 4, one-sixth of the basal-NSC-medium was supplemented. The incubation was extended until day 6, and neurospheres were collected, mechanically dispersed into single cells, and then cultured as described above for passaging.

To induce NSC differentiation, the dissociated cells were seeded onto 200 μg/mL poly-L-lysine-coated coverslips at a density of 5×10^4 cells/coverslip. The growth factors were removed from the growth medium, and then either 1% or 10% FBS (GIBCO) was added. The cultures were allowed to differentiate for 5 days *in vitro* before RT-PCR, Western blot, or immunofluorescence analyses.

RT-PCR was used to detect the mRNA expressions of BMP-2 and BMP-4. Briefly, total RNAs, from NSCs and their derivatives under different differentiation conditions, were extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed to cDNA, and PCR was performed by a routine method (Lu et al. 2008b, Lu et al. 2010). PCR products were analyzed on 1% agarose gel. β-actin was used as an internal control. The sequences of specific primers for RT-PCR are given in Table I.

Western blot analysis was used to detect the protein expressions of BMP-2 and BMP-4. Briefly, cells were washed twice with PBS and lysed in RIPA buffer [50 mM Tris–HCl, 150 mM NaCl, 1.0 mM Na_3VO_4, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 30 μl/ml aprotinin and 4 μg/ml leupeptin, pH 7.5]. The supernatant was clarified by centrifugation at 16 000 g for 10 min at 4°C. The protein concentrations of the lysate were determined using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). For Western blotting, supernatants were diluted in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol,

50 mM DTT and 0.1% bromophenol blue) and boiled for 5 min. Equal amounts of protein (20 μg) were resolved on 12% SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked at room temperature (RT) for 1 h in 5% (w/v) dry skim milk in TBS plus 0.1% Tween-20 (TBST), rinsed in TBST and incubated with primary antibodies at 4°C overnight. Primary antibodies used were mouse anti-β-actin (1:5000, Santa Cruz Biotechnology) and goat anti-BMP-2 and BMP-4 antibody (1:400, Santa Cruz Biotechnology). After being rinsed with TBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody (all from KPL, Gaithersburg, MD, USA) for 1 h at RT. To visualize the immunoreactive proteins, the ECL kit (Pierce) was used following the manufacturer's instructions. Films were digitized and densitometry was performed using Gel-Pro analyzer (Media Cybernetics, Silver Spring, MD).

Immunofluorescence double staining was used to detect cellular localization of BMP-2 and BMP-4. For NSCs, free-floating spheres were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, washed in phosphate-buffered saline (PBS), and cryoprotected in PBS containing 30% sucrose. Spheres were embedded in OCT (Sakura FineTec Inc., Torrance, CA) and sectioned with a cryostat. For differentiated cells, the cells were fixed with 4% PFA in PBS (0.01 M, pH 7.4) for 10 min at room temperature (RT). Sections of neurospheres or differentiated cells mounted on poly-L-ornithine-coated coverslips were blocked with 10% normal goat serum (NGS) containing 0.3% Triton X-100 for 1 h at room temperature (RT), and incubated with one of the monoclonal mouse anti-rat primary antibodies against nestin (1:100; Pharmingen, San Diego, CA) for NPCs, βIII-tubulin (1:800, Sigma) for neurons, receptor interacting protein (RIP, 1:2000, Chemicon, Temecula, CA) for oligodendrocytes, glial fibrillary
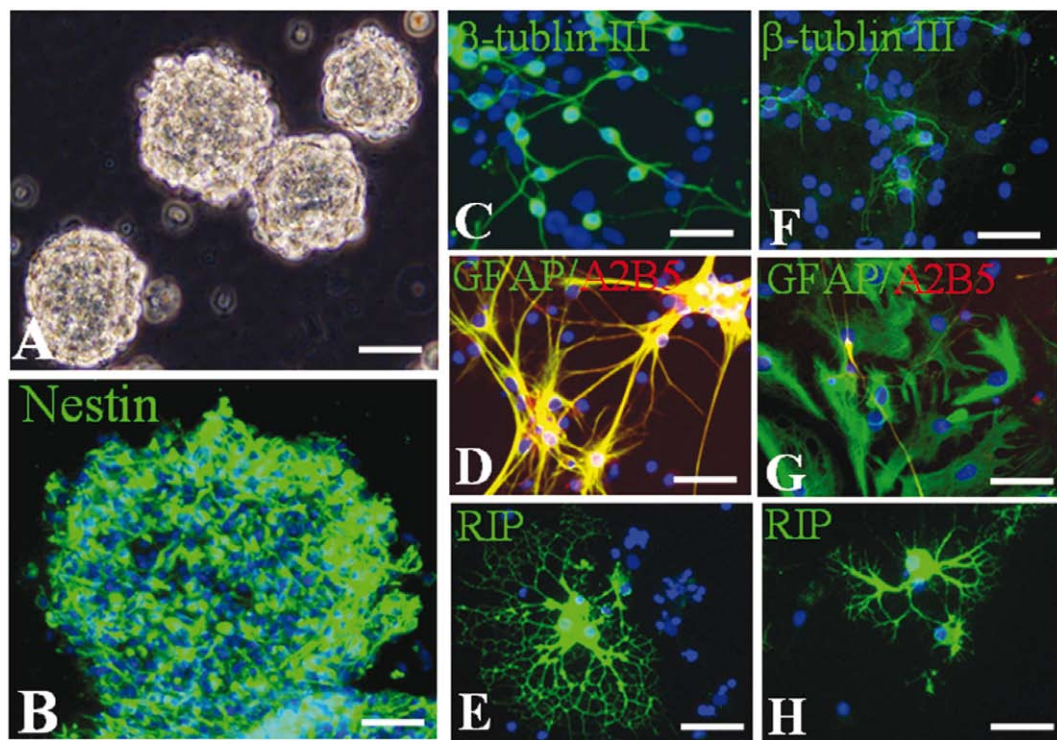

Fig. 1. Morphological and immunocytochemical characteristics of NSCs and their derivatives under different differentiation conditions. (A) Phase-contrast photomicrograph of neurospheres cultured in growth medium supplemented with 20 ng/ml EGF and 20 ng/ml bFGF. (B) Cells in a neurosphere were immunopositive for nestin (green). (C–E) When cultured in medium containing no growth factors but 1% FBS for 5 days, NSCs differentiated into neurons (βIII-tubulin$^+$, C), type-2 astrocytes (GFAP$^+$A2B5$^+$, D) and oligodendrocytes (RIP$^+$, E). (F–H) When cultured in medium containing no growth factors but 10% FBS for 5 days, NSCs differentiated into neurons (βIII-tubulin$^+$, F), type-1 astrocytes (GFAP$^+$A2B5$^-$, G) and oligodendrocytes (RIP$^+$, H). Cells in B–H were counterstained with Hoechst 33342 (blue), a nuclear dye. Scale bars are: 100 μm in A, 50 μm in B, 25 μm in C–H.

acidic protein (GFAP, 1:200, Sigma) for astrocytes, A5B5 (IgM, 1:100, R&D, Minneapolis, MN) for type-2 astrocytes and the polyclonal goat anti-rat BMP-2 and BMP-4 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for BMP-2 and BMP-4, overnight at 4°C. The slides or coverslips were then incubated with FITC-conjugated goat anti-mouse IgG (1:120, Sigma) and Rhodamine-conjugated rabbit anti-goat IgG (1:200, Santa Cruz Biotechnology) or Rhodamine-conjugated goat anti-mouse IgM (1:200, Santa Cruz Biotechnology) for 1 h at 37°C. The slides or coverslips were rinsed and mounted with Gel/Mount aqueous mounting media (Biomeda Corp, Foster City, CA) containing Hoechst 33342, a nuclear dye (0.5 μM, Sigma). The results of the immunostaining were examined with an Olympus BX60 microscope. The data were analyzed by Student's t-test to determine whether there were significant differences between individual groups. All differences were considered significant at $P<0.05$.

After dissociated NSC cells were plated, these single cells proliferated to form small clusters and then larger spheres (neurospheres) floating within the growth medium in the presence of EGF and bFGF within 3–4 days (Fig. 1A). Immunostaining of the sectioned neurospheres revealed that the cells within the sphere were positive for nestin (Fig. 1B), an intermediate filament protein expressed mainly by stem or precursor cells. When induced to differentiate in different media for 5 days, the cells differentiated into a mixture of neurons (βIII-tubulin+, 16.58% ± 5.23%; Fig. 1C), type-2 astrocytes (GFAP+A2B5+, 58.58% ± 6.74%; Fig.1D), and oligodendrocytes (RIP+, 16.47% ± 5.88%; Fig. 1F) in medium containing 1% fetal bovine serum (FBS). However, in medium containing 10% FBS, the cells mostly differentiated into type-1 astrocytes (GFAP+A2B5-, 87.56% ± 4.68%), and the proportion of neurons and oligodendrocytes were relatively decreased. The βIII-tubulin+ and RIP+ cells were 5.24% ± 2.28% and 6.88% ± 3.12%, respectively (Fig. 1F–H).

The RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B) showed that in the growth medium, the NSCs showed no detectable mRNA and protein expression of BMP-2 and BMP-4. After the growth factors were removed from the growth medium, and 1% or 10% FBS were added, the mRNA and protein expression of BMP-2 and BMP-4 could be detected under both conditions. In Western blot analysis, two immunoreactive bands, one at ~18 kDa (the monomeric form) and the other at ~36 kDa (the dimeric form) were detected for BMP-2. A single immunoreactive band at ~36 kDa was detected for BMP-4, probably representing a dimeric conformation of the mature peptide (Garimella et al. 2008). These results showed that BMP-2 and BMP-4 mainly express in the derivatives of NSCs, but not in NSCs.

Immunofluorescence double staining showed that both BMP-2 and BMP-4 were mainly expressed in both type-1 and type-2 astrocytes (Fig. 3C–F), but not in NSCs (Fig. 3A–B), oligodendrocyte (Fig. 3G–J) and neurons (Fig. 3K–N). The expression pattern of both BMP-2 and BMP-4 in all kinds of NSC derivatives had no significant difference between 1% and 10% FBS containing differentiation conditions ($P>0.05$, Fig. 3O)
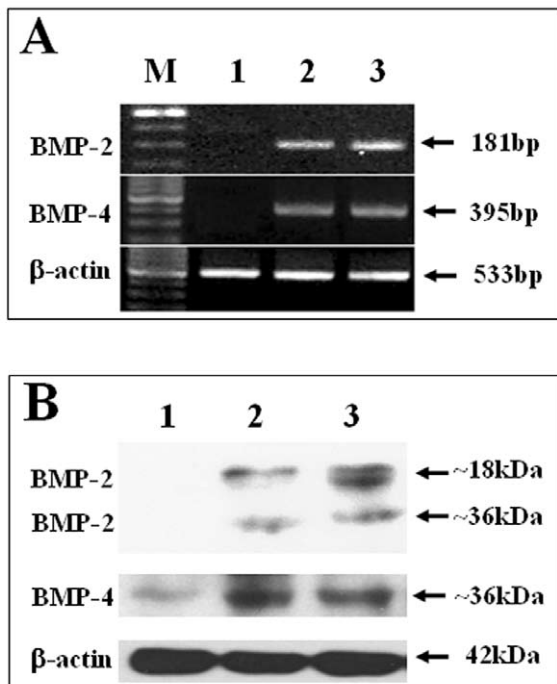

Fig. 2. BMP-2 and BMP-4 mRNA and protein expression in NSCs and their derivatives under different differentiation conditions. (A) Agarose gel electrophoresis of RT-PCR products generated by specific primers for BMP-2, BMP-4 and β-actin. (B) Expressions of BMP-2 and BMP-4 protein in different cells detected by Western blot. (M) 100 bp DNA ladder; (1) NSCs cultured in growth medium supplemented with 20 ng/ml EGF and 20 ng/ml bFGF; (2) Type-2 astrocytes induced from NSCs in medium containing no growth factors but 1% FBS for 5 days; (3) Type-1 astrocytes induced from NSCs in medium containing no growth factors but 10% FBS for 5 days.

BMPs, a subgroup of the TGF-β superfamily, play critical roles in neural progenitor cell fate determination (Mehler et al. 1997, Dang and Tropepe 2006, Sabo et al. 2009). It has been reported that when embryonic neural progenitor cells in culture were exposed to BMPs, their developmental fate was altered from neuronal to astrocytic cells (Gross et al. 1996, Nakashima et al. 2001). Several members of the BMP family, such as BMP-2 and BMP-4, have also been implicated as repressors of oligodendrocyte development *in vitro* by shifting oligodendrocyte precursors into the astrocyte lineage (See et al. 2004). Although the roles of BMPs in neural progenitor cell developmental fate have been extensively studied *in vitro*, most of these results are
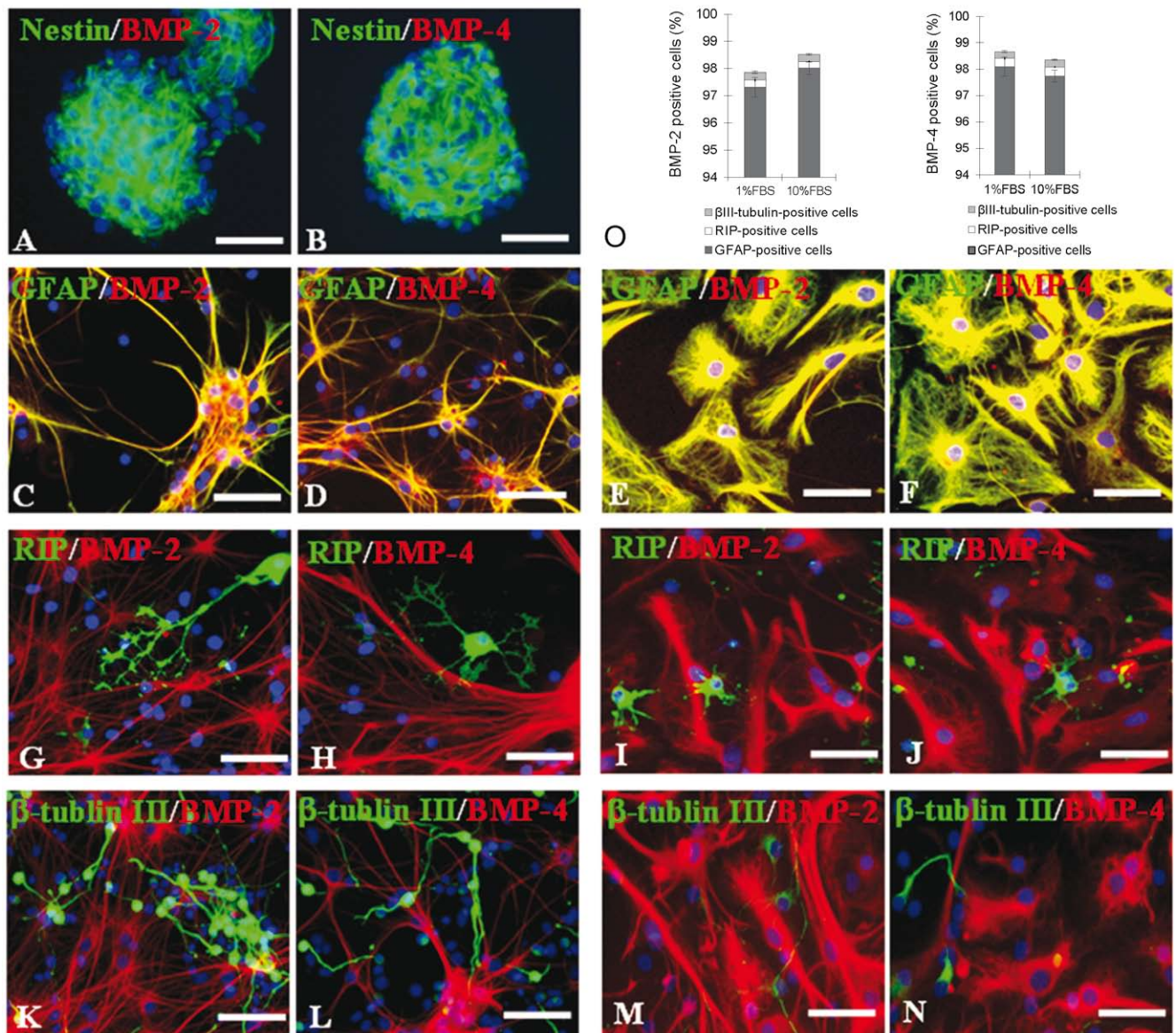

Fig. 3 Immunostaining of different cell type markers and BMPs. (A–B) Cells in a neurosphere section were immuno-positive for nestin (green), and immuno-negative for BMP-2/4 (red). (C–N) When cultured in medium containing no growth factors but 1% or 10% FBS for 5 days, Colocalization of cell specific markers (green) and BMP-2 or BMP-4 (red) in NSCs and their lineages were detected by immunofluorescence double staining. Note that BMP-2 and BMP-4 were co-localized in both type-1 and type-2 astrocytes (C–F), but not in oligodendrocytes (G–J) and neurons (K–N), which can be observed in the merge of double exposures for each. Cells were counterstained with Hoechst 33342 (blue), a nuclear dye. Scale bars: 25μm. (O) The statistical graphs showed the expression pattern of BMP-2 and BMP-4 in neural stem cells and their derivatives under different differentiation conditions. Data are given as means ± SD, $n=4$, each $P>0.05$

acquired by using the recombinant BMPs. Therefore, little information is available for endogenous expression of BMPs in neural progenitor cells and their derivatives under culture conditions.

In this study, we used an *in vitro* model with NSCs generated from rat embryonic spinal cord that could be induced to differentiate into different neural cell lineages under different culture conditions. At 5 days *in vitro*, three types of cells (neurons, oligodendrocytes, and astrocytes) differentiated from NSCs were observed in media containing no growth factors but 1% or 10% FBS. This indicated the NSCs have the capacity to differentiate into neurons and glial cells under both conditions. However, the differentiated astrocytes displayed two distinct morphological characteristics: (1) flat cell bodies with rich cytoplasm, large nuclei, GFAP-positive and A2B5-negative which resemble type-1 astrocytes under 10% FBS condition, and (2) a more satellite morphology with long filiformed processes and GFAP/A2B5-double positive which resemble type-2 astrocytes under 1% FBS condition. This is consistent with previous reports (Fu et al. 2005) and could be taken as a model to study the BMP expression in NSCs and their derivatives.

Based on the above NSC differentiation model, we first detected BMP-2 and BMP-4 mRNA expressions in NSCs and their derivatives. We found that there were no detectable BMP-2 and BMP-4 mRNA expressions in NSCs in the growth medium with EGF and bFGF. However, after the growth factors were removed from the growth medium, and 1% or 10%FBS was added, the mRNA and protein expression of BMP-2 and BMP-4 could be detected under both conditions. To verify the RT-PCR results, we used Western blot analysis to detect BMP-2 and BMP-4 protein expression. Using different antibodies which can recognize BMP-2 and BMP-4, respectively, we found two immunoreactive bands for BMP-2, one at ~18 kDa (the monomeric form) and the other at ~36 kDa (the dimeric form). A single immunoreactive band at ~36 kDa was detected for BMP-4, which probably represents a dimeric conformation of the mature peptidethe expression (Garimella et al. 2008). In order to further determine the cellular distribution of BMP-2 and BMP-4 in neural stem cells and their derivatives under different differentiation conditions, immunofluorescence double staining was used. Finally, we found that both BMP-2 and BMP-4 were mainly expressed in both type-1 and type-2 astrocytes, but not in NSCs, oligodendrocytes and neurons.

In conclusion, the present study demonstrated that BMP-2 and BMP-4 were constitutively expressed in both types I and II astrocytes differentiated from NSCs under different differentiation conditions *in vitro*. This suggested that the astrocytes might be one source of BMPs during the differentiation of NSCs. However, in our model, we can not exclude the possibility that microglia or endothelial cells could also be a source of BMPs.

Abematsu M, Smith I, Nakashima K (2006) Mechanisms of neural stem cell fate determination: extracellular cues and intracellular programs. Curr Stem Cell Res Ther 1: 267–277.

Dang L, Tropepe V (2006) Neural induction and neural stem cell development. Regen Med 1: 635–652.

Fu SL, Ma ZW, Yin L, Iannotti C, Lu PH, Xu XM (2005) Region-specific growth properties and trophic requirements of brain- and spinal cord-derived rat embryonic neural precursor cells. Neuroscience 135: 851–862.

Garimella R, Tague SE, Zhang J, Belibi F, Nahar N, Sun BH, Insogna K, Wang J, Anderson HC (2008) Expression and synthesis of bone morphogenetic proteins by osteoclasts: a possible path to anabolic bone remodeling. J Histochem Cytochem 56: 569–577.

Gross RE, Mehler MF, Mabie PC, Zang Z, Santschi L, Kessler JA (1996) Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron 17: 595–606.

Hu JG, Fu SL, Zhang KH, Li Y, Yin L, Lu PH, Xu XM (2004) Differential gene expression in neural stem cells and oligodendrocyte precursor cells: a cDNA microarray analysis. J Neurosci Res 78: 637–646.

Lu HZ, Hu JG (2009) Expression of bone morphogenetic proteins-2/4 in neural stem cells and their lineages. Acta Neurobiol Exp (Wars) 69: 441–447.

Lu HZ, Wang YX, Li Y, Fu SL, Hang Q, Lu PH (2008a) Proliferation and differentiation of oligodendrocyte progenitor cells induced from rat embryonic neural precursor cells followed by flow cytometry. Cytometry A 73: 754–760.

Lu HZ, Wang YX, Zou J, Li Y, Fu SL, Jin JQ, Hu JG, Lu PH (2010) Differentiation of neural precursor cell-derived oligodendrocyte progenitor cells following transplantation into normal and injured spinal cords. Differentiation 80: 228–240.

Lu HZ, Xu L, Zou J, Wang YX, Ma ZW, Xu XM, Lu PH (2008b) Effects of autoimmunity on recovery of function in adult rats following spinal cord injury. Brain Behav Immun 22: 1217–1230.

Mehler MF, Mabie PC, Zhang D, Kessler JA (1997) Bone morphogenetic proteins in the nervous system. Trends Neurosci 20: 309–317.

Nakashima K, Takizawa T, Ochiai W, Yanagisawa M, Hisatsune T, Nakafuku M, Miyazono K, Kishimoto T, Kageyama R, Taga T (2001) BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. Proc Natl Acad Sci U S A 98: 5868–5873.

Raff MC (1989) Glial cell diversification in the rat optic nerve. Science 243: 1450–1455.

Raff MC, Abney ER, Cohen J, Lindsay R, Noble M (1983) Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. J Neurosci 3: 1289–1300.

Sabo JK, Kilpatrick TJ, Cate HS (2009) Effects of bone morphogenic proteins on neural precursor cells and regulation during central nervous system injury. Neurosignals 17: 255–264.

See J, Zhang X, Eraydin N, Mun SB, Mamontov P, Golden JA, Grinspan JB (2004) Oligodendrocyte maturation is inhibited by bone morphogenetic protein. Mol Cell Neurosci 26: 481–492.