Expression of bone morphogenetic proteins-2/4 in neural stem cells and their lineages

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Neural stem cells (NSCs) are multipotent progenitor cells that possess the ability to self-renew and generate different neural cell types. Bone morphogenetic proteins (BMPs) play critical roles in the determination of the fate of NSCs. Although some reports delineate BMP expression in the early developing central nervous system (CNS), they deal mainly with its expressions in restricted areas. However, little information is available for BMP expression in NSCs and their lineages. In this study, based on an *in vitro* model in which NSCs can self-renew and differentiate into astrocytes, neurons or oligodendrocytes, we mimicked the environment of NSC development *in vivo*. Using an antibody which can recognize BMP-2 and BMP-4, we showed that BMP-2/4 is only expressed in astrocytes. Inflammatory cytokines (tumor necrosis factor-α and/or interferon-γ) do not influence the expression pattern of BMPs in NSCs and their lineages. These results suggest that astrocytes might be one source of BMPs during the differentiation of NSCs and in the inflammatory environment after CNS injury.

Key words: neural stem cells, bone morphogenetic proteins, differentiation, inflammatory factors, astrocytes

INTRODUCTION

Neural stem cells (NSCs) are multipotent progenitor cells present within the germinal zones of the central nervous system (CNS) throughout embryonic, postnatal and adult life. They possess the ability to self-renew and generate different neural cell types within discrete temporospatial developmental windows when they encounter different signals. Recent studies have provided evidence that soluble protein mediators such as cytokines and transcription factors play critical roles in NSC fate determination (Abematsu et al. 2006). These mediators, bone morphogenetic proteins (BMPs), a subgroup of the TGF-β superfam-

ily, can promote the differentiation of neural progenitor cells into astrocytes and inhibit oligodendrogliogenesis (Mabie et al. 1997, Mehler et al. 2000). The inflammatory factors also influence NSC differentiation. A previous report indicated that differentiation of engrafted NSCs in injured CNS is restricted towards the astrocytic lineage because of the inflammatory environment (Ricci-Vitiani et al. 2006). However, another report found that inflammatory cytokines only induce apoptotic cell death but have no effects on NSC lineage fate (Ben-Hur et al. 2003, Feldhaus et al. 2004).

In the early developing CNS, BMPs are expressed particularly at the lateral edges of the neural plate and subsequently in the dorsal midline of the neural tube, and regulate production of types of dorsal neural cells (Liem et al. 1995, Furuta et al. 1997, Chen and Panchision 2007). In adult CNS, BMP-4 is found to be

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widely expressed throughout the CNS, mainly in neurons and astrocytes (Mikawa et al. 2006). *In situ* hybridization has indicated that expression of BMP-7 mRNA is present only in glial cells in uninjured spinal cord. After injury, the number of BMP-7-expressing glial cells is increased, BMP-7 expression also become apparent in motor neurons (Setoguchi et al. 2001). BMP-6 is expressed in neurons of the hippocampus and cortex in normal adult rat brains. A pronounced expression of BMP-6 in astroglia located to a lesion becomes obvious 48 h postinjury (Zhang et al. 2006). These studies suggest that the inflammatory environment might influence the expression pattern of BMPs in the NSCs and their lineages.

In general, NSCs can be isolated from the brain and spinal cord of embryonic, neonatal or adult animals and proliferated and differentiated under special conditions *in vitro*. This provides a powerful tool for the study of lineage segregation of NSCs. In this study, we detected the expression of BMP-2/4 (the antibody cannot distinguish between these homologues of the decapentaplegic family) in embryonic spinal cord-derived NSCs and their lineages. We found that BMP-2/4 is only expressed in astrocytes, and inflammatory cytokines do not influence the expression pattern of BMPs in the NSCs and their lineages.

METHODS

Culture of spinal cord-derived NSCs

Spinal cord-derived NSCs were prepared as described previously (Fu et al. 2007, Lu et al. 2008). 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, NY). 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 passaged.

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. Then growth factors were removed from the growth medium, and 1% fetal bovine serum (FBS, GIBCO) was added (referred to as NSC-differentiation-medium). The cultures were allowed to differentiate for 5 days *in vitro* before being fixed for immunostaining.

NSCs and their lineages treatment with inflammatory factors

The dissociated NSCs were passaged in basal-NPC-medium, or differentiated in NSC-differentiation-medium as noted above. To treat with inflammatory factors, the cells were incubated with different concentrations of recombinant rat tumor necrosis factor-α (TNF-α, 0, 25, 50, and 100 U/ml) or interferon-γ (IFN-γ, 0, 250, 500, and 1 000 U/ml) or a combination (both from PeproTech EC, London, UK) for 5 days for assessing their differentiation and BMP-2/4 expression.

Immunofluorescence staining

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, glial fibrillary acidic protein (GFAP, 1:200, Sigma) for astrocytes, and receptor interacting protein (RIP, 1:2 000, Chemicon, Temecula, CA) for oligodendrocytes, and the polyclonal rabbit anti-rat BMP-2/4 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for BMP-2/4,

overnight at 4°C. The slides or coverslips were then incubated with FITC-conjugated goat anti-mouse IgG (1:120, Sigma) and Rhodamine-conjugated goat anti-rabbit IgG (1:50, Cappel, Costa Mesa, CA) for 1 h at 37°C. The slides or coverslips were rinsed and mount-ed 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.
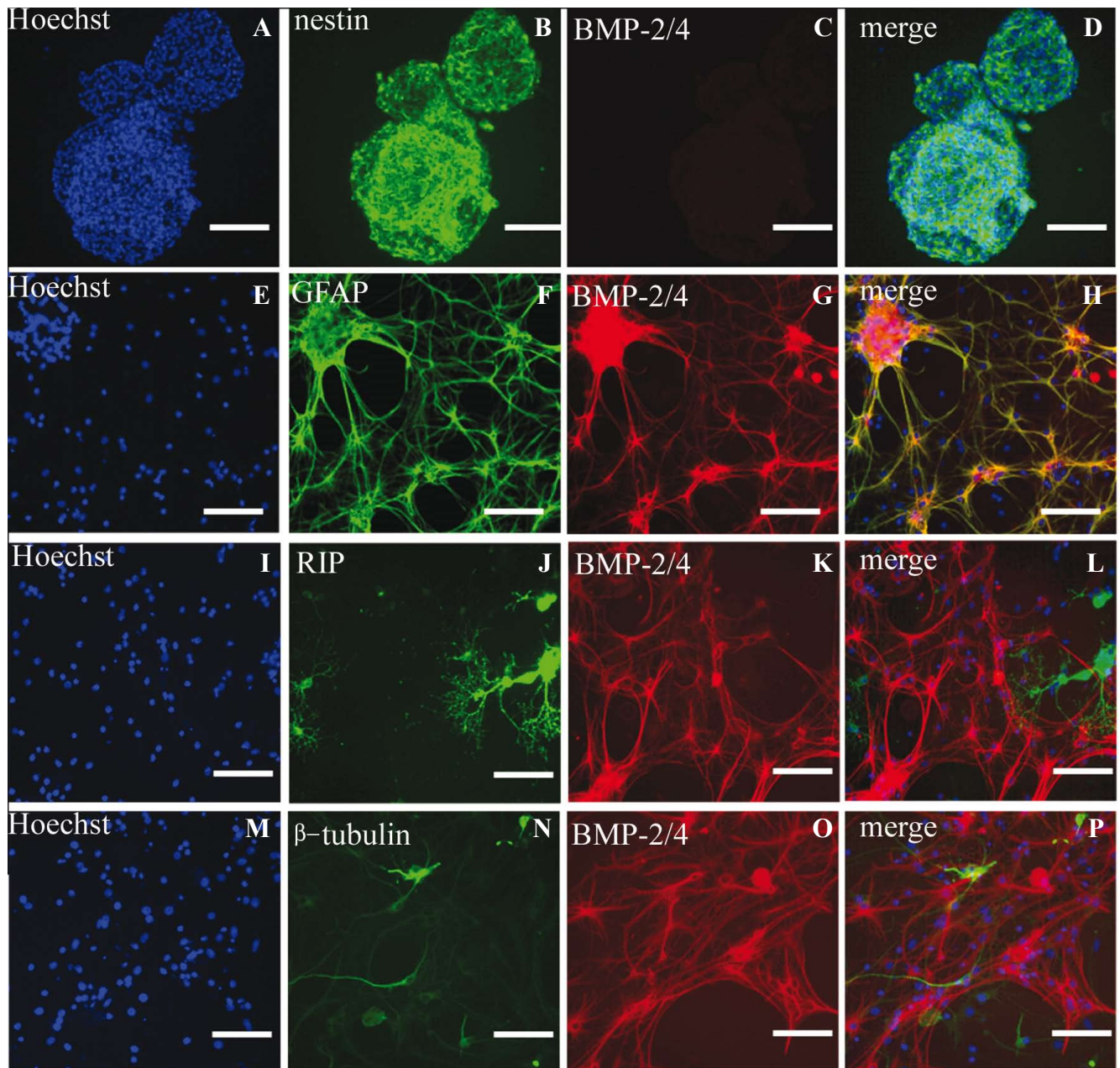

Fig. 1. The characteristics of NSCs and expression of BMP-2/4 in NSCs and their lineages *in vitro*. (A)–(D) Cells in a neu-rosphere section were immuno-positive for nestin (green), and immuno-negative for BMP-2/4 (red). (E)–(P) When cultured in medium containing no growth factors but 1% FBS for 5 days, NSCs differentiated into astrocytes (GFAP+, F), oligoden-drocytes (RIP+, J) and neurons (βIII-tubulin+, N). Colocalization of cell specific markers (green) and BMP-2/4 (red) in NSCs and their lineages were detected by immunofluorescence double staining. Note that BMP-2/4 were colocalized in nearly all astrocytes (H), but not in oligodendrocytes (L) and neurons (P), which can be appreciated in the merge of double exposures for each. Cells were counterstained with Hoechst 33342 (blue), a nuclear dye. Scale bars are 50 µm.

Statistical analysis

The data were analyzed by one way ANOVA followed by Student-Newman-Keuls tests of multiple comparisons to determine whether there were signifi-

cant differences between individual groups. All differences were considered significant at $P<0.05$.

RESULTS

Isolation, Induction, and Identification of NSCs

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. 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 (Lendahl et al. 1990). When plated onto poly-ornithine-coated coverslips in NSC-differentiation-medium for 5 days, cells migrated out of the spheres and differentiated into a mixture of astrocytes (GFAP$^+$, 60.24% ± 7.65%; Fig.1F), oligodendrocytes (RIP$^+$, 16.47% ± 5.88%; Fig.1J), and neurons (βIII-tubulin$^+$, 14.54% ± 4.98%; Fig.1N).

Expression of BMP-2/4 in neural stem cells and their lineages

Immunofluorescence double staining was used to detect the expression of BMP-2/4 in neural stem cells and their lineages. The results showed that BMP-2/4 mainly express in GFAP positive astrocytes (Fig. 1E–H), but not in NSCs (Fig. 1A–D), oligodendrocyte (Fig. 1I–L) and neurons (Fig. 1M–P).

Effect of inflammatory factors on the lineage fate of NSCs and expression of BMP-2/4 in neural stem cells and their lineages

To assess the effect of inflammatory factors on the differentiation potential of NSCs and BMP-2/4 expression in NSCs and their lineages, NSCs were cultured for 5 days in basal-NSC-medium or NSC-differentiation-medium with or without TNF-α and/or interferon-γ. The results showed that even though in the combination group with the maximum concentration of inflammatory factors, there were no effects on the differentiation potential of NSCs (Fig 2A) and the expression of BMP-2/4 in nestin$^+$, βIII-tubulin$^+$, RIP$^+$ or GFAP$^+$ cells (Fig 2B), as compared to the respective non-treated controls.
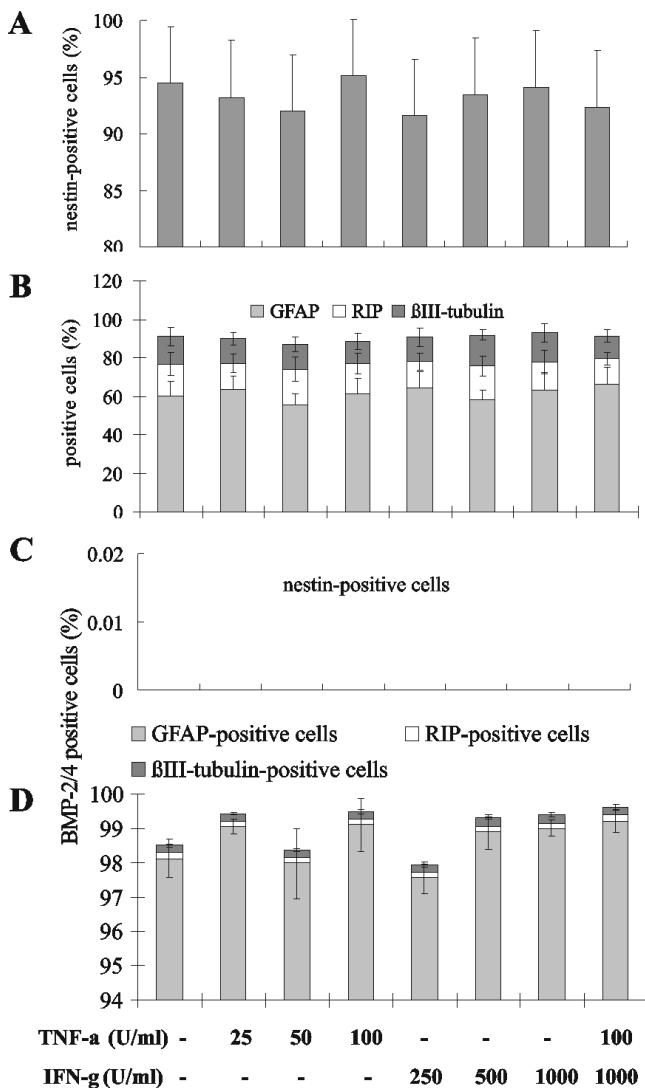

Fig. 2. Effect of inflammatory factors on the lineage fate of NSCs and expression of BMP-2/4 in NSCs and their lineages. NSCs were cultured for 5 days in basal-NSC-medium or NSC-differentiation-medium added to different concentrations of TNF-α or/and IFN-γ as described in the Methods. (A) The statistical graphs show that the treatment of NSCs with TNF-α or/and IFN-γ did not result in a substantial change in the percentage of nestin$^+$ (A), GFAP$^+$, βIII-tubulin$^+$ and RIP$^+$ Cells (B), and the expression of BMP-2/4 in nestin$^+$ (C), GFAP$^+$, βIII-tubulin$^+$ and Rip$^+$ Cells (D), as compared to the respective non-treated controls. Data are given as means ± SD, n=4, each $P>0.05$.

DISCUSSION

The BMPs can be broadly classified into three sub-families. In the first group, consisting of BMP-2 and BMP-4, which have an 80% amino acid sequence homology. In the second group are BMP-5, -6 and -7, which have a 78% amino acid sequence homology. The third group, composed solely of BMP-3, is significantly different from the other members of the BMP family and generally stands alone (Rengachary 2002). It is of interest that both BMP-2 and BMP-4 are closely related by their amino acid sequence and act on the same receptor, implying that these two BMPs might have similar biological functions (Rengachary 2002). Previous reports showed that BMPs are expressed particularly at the lateral edges of the neural plate and subsequently in the dorsal midline of the neural tube in the early developing CNS (Liem et al. 1995, Furuta et al. 1997, Chen and Panchision 2007). However, little information is available for BMPs expression in the NSCs and their lineages

This study was based on an *in vitro* model that NSCs generated from rat embryonic spinal cord could be induced to differentiate into different neural cell lineages and that we could mimic the environment of NSCs development *in vivo*. Using an antibody which can recognize BMP-2 and BMP-4, we showed that BMP-2/4 is only expressed in astrocytes, while not in NSCs, neurons and oligodendrocytes. These findings demonstrate that the astrocytes might be one source of BMPs during the differentiation of NSCs.

After injury to the adult CNS, one of the earliest responses to injury is the infiltration of macrophages and the activation of microglia, which starts a cytokine/ growth factor cascade (Giulian et al. 1989). Numerous growth factors and cytokines are released that could potentially regulate the expression of BMPs. It has been shown that NSCs express receptors for TNF-α and IFN-γ, which might influence the growth and differentiation of NSCs (Deleyrolle et al. 2006). It has also been found that some BMP expression is upregulated in response to CNS injury and is suggested to be induced, directly or indirectly, by inflammatory cytokines released after injury (Setoguchi et al. 2001, Zhang et al. 2006). These results suggest that the expression of BMPs might be regulated by inflammatory cytokines present in injured CNS environments. Previous studies have revealed that TNF-α and IFN-γ are increased after traumatic brain injury (Fan et al. 1996) and spinal cord injury (Hayashi et al. 2000, Yan et al. 2001,

Nakamura et al. 2003). Here, we mimicked the inflammatory environment of NSCs development *in vivo* by adding these two inflammatory cytokines to growth or differentiation medium of NSCs. We found that both TNF-α and IFN-γ can upregulate the expression of BMP-2/4 in astrocytes (data not show). This result is in agreement with the phenomenon that BMPs are upregulated in the injured CNS where mass astrocytes are activated and proliferated (Setoguchi et al. 2001, Fuller et al. 2007, Hampton et al. 2007, Matsuura et al. 2008). Nevertheless we did not find an effect on the differentiation potential of NSCs after adding both factors in combination or alone, which was consistent with the previous reports (Ben-Hur et al. 2003, Feldhaus et al. 2004). Another important result was that the inflammatory factors also have no effect on expression pattern of BMPs in the NSCs and their lineages. This seems to be paradoxical given the results of other researchers who found that the BMPs can be expressed in cells besides astrocytes in injured CNS (Setoguchi et al. 2001, Mikawa et al. 2006, Zhang et al. 2006). The explanation for this unexpected result might be that TNF-α and/or IFN-γ probably do not fully reflect the complex inflammatory situation after CNS trauma characterized by massive cell death. It is possible that the changed expression pattern of BMPs in injured CNS is an indirect consequence induced by inflammatory cytokines. Certainly, the situation *in vivo* may be more complex and the factors which influence the expression pattern of BMPs in different cell types in the CNS remain to be determined.

CONCLUSION

The present study demonstrates that BMP-2/4 is constitutively expressed in astrocyte lineages differentiated from NSCs, and inflammatory cytokines TNF-α and IFN-γ do not influence the expression pattern of BMP-2/4 in the NSCs and their lineages *in vitro*. These results suggest that the astrocytes might be one source of BMPs during the differentiation of NSCs and in the inflammatory environment after CNS injury.

ACKNOWLEDGMENT

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