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BONE MORPHOGENETIC PROTEIN 4 STIMULATES ATTACHMENT OF NEUROSPHERES AND ASTROGENESIS OF NEURAL STEM CELLS IN NEUROSPHERES VIA PHOSPHATIDYLINOSITOL 3 KINASE-MEDIATED UPREGULATION OF N-CADHERIN

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Abstract—The neurosphere culture system is useful for expanding neural stem cells (NSCs) without affecting self-renewal potential and multipotency. However, the extrinsic signals that affect the formation or dissociation of neurospheres are poorly understood. Here, we found that bone morphogenetic protein 4 (BMP4) induced the attachment of neurospheres, astrocytic differentiation, and migration of neurosphere NSCs. These outcomes were accompanied by Akt activation and upregulation of the adhesion molecule, N-cadherin. A phosphatidylinositol 3 kinase (PI3 kinase) inhibitor (LY294002) blocked attachment of neurosphere, astrocytic differentiation, migration, and N-cadherin upregulation of neurosphre NSCs. The PI3 kinase-Akt pathway appeared to selectively mediate the effects of BMP4, as neurosphere attachment was unaffected by MEK inhibitors (PD98059 and U0126). Importantly, a neutralizing N-cadherin antibody inhibited BMP4-induced neurosphere attachment, astrocytic differentiation, and migration of neurosphere NSCs. Together, these findings show that BMP4-induced attachment of neurospheres is related to the astrocytic differentiation of these cells and that these effects are attributable, at least in part, to PI3 kinase-Akt pathway-dependent induction of N-cadherin.

Key words: attachment, TGFβ family, Akt pathway, cell adhesion molecules, central nervous system.

Neurospheres are three-dimensional, free-floating aggregates of neural stem cells (NSCs) (Singec et al., 2006). These structures have been used as culture systems for the expansion and differentiation of NSCs (Shanley and Sullivan, 2006). They have also been transplanted into animals to replace injured or dysfunctional tissues or organs (Fricker et al., 1999; Eriksson et al., 2003). In fact, neurosphere transplantation has been attempted for the treatment of neurodegenerative diseases such as Parkinson's disease (Meissner et al., 2005). Neurospheres are produced using variable-stage NSCs from the CNS, which are expanded using mitogens such as epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) (Singec et al., 2006). The mitogen-responsive cells can be passaged at least 10 times to produce additional neurospheres without significantly affecting proliferation or differentiation potential (Reynolds and Weiss, 1996; Reynolds and Rietze, 2005). The ability of NSCs to form neurospheres is an important indicator of stem cell characteristics such as self-renewal and the potential to differentiate into neurons, astrocytes, or oligodendrocytes (Reynolds and Weiss, 1996). However, the extrinsic signals that affect formation or dissociation of the neurosphere are poorly understood.

Bone morphogenetic proteins (BMPs) are a family of multi-functional growth factors that include transforming growth factor-β (TGF-β). BMPs are secreted proteins that participate in various biological processes, including neural tube patterning, mesoderm differentiation (Attisano and Wrana, 2002), as well as regulation of proliferation and differentiation of many different cell types (Massague and Chen, 2000). Importantly, BMPs play key roles in the formation and development of the nervous system (Liu and Niswander, 2005) and regulation of stem cell fate commitment (Varga and Wrana, 2005). Bone morphogenetic protein 4 (BMP4) in particular is known to participate in the astrocytic differentiation of neural progenitor/stem cells (NPCs/NSCs) (Gross et al., 1996; Bonaguidi et al., 2005; Fukuda and Taga, 2005; Weible and Chan-Ling, 2007). BMP4 can also induce neuronal differentiation (Varley and Maxwell, 1996; Li et al., 1998; Liu and Niswander, 2005), depending on the balance between neuronal and astrocytic differentiation pathways (Fukuda and Taga, 2005). The adherence of cells is important for differentiation as well as for proliferation, migration, neurite outgrowth, and synaptogenesis (Derycke and Bracke, 2004). One of the major molecules that mediates cell attachment is N-cadherin (Derycke and Bracke, 2004). This membrane protein plays important roles in proliferation, migration, and invasion in cancerous cell types (Lambert et al., 2000; Li et al., 2001). It also controls cell-cell contact and cell-matrix adhesion during angiogenesis and neurite outgrowth (Utton et al., 2001). Here, we provide evidence that BMP4 stimulates the adhesion, astrocytic differentiation, and migration of neuro-

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Abbreviations: bFGF, basic fibroblast growth factor; BMPs, bone morphogenetic proteins; BMP4, bone morphogenetic protein 4; DAPI, 4',6-diamidino-2-phenylindole; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FACS, fluorescence activated cell sorting; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NPC, neural progenitor cell; NSC, neural stem cell; p-Akt, phosphorylated Akt; PBS, phosphate-buffered saline; PI3 kinase, phosphatidylinositol 3 kinase; RT, room temperature; SDS, sodium dodecyl sulfate; TGFβ, transforming growth factor-β; Tuj1, neuron-specific class III beta-tubulin.

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sphere NSCs through phosphatidylinositol 3 (PI3) kinase pathway-dependent induction of N-cadherin.

EXPERIMENTAL PROCEDURES

Neurosphere cultures

NSCs were isolated from the subventricular zone of brains collected from Sprague–Dawley rat embryos at E14.5 (KOATECH, Gyeonggi Do, Korea). All animal procedures were approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. The cells were mechanically dissociated from the tissues and grown in uncoated 6-well plates (Techno Plastic Products, St. Louis, MO, USA) at a concentration of 1×10^5 cells/ml. NPCs were grown in N2 medium [Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (Invitrogen, Carlsbad, CA, USA)] containing 100 μM putrescine, 30 nM selenite, 20 nM progesterone, 1.55 mg/ml D-(+)-glucose, 25 μg/ml insulin, 0.1 μg/ml apo-transferrin (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mM glutamax, 100 IU/ml penicillin, and 100 μg/ml streptomycin supplemented with 10 ng/ml bFGF (Peprotech, Princeton, NJ, USA) and 20 ng/ml human EGF. The neurospheres, which formed after 3–4 days, were harvested by centrifugation, enzymatically dissociated with trypsin-EDTA (Invitrogen), and replated in 6-well plates for maintenance or 24-well plates for immunocytochemistry. Two days later, neurospheres were treated for 1 h with 100 ng/ml Noggin (Peprotech), 10 μM LY294002, 10 μM PD98059, 5 μM U0126 (AG. Scientific Inc., San Diego, CA, USA), or N-cadherin/A-CAM monoclonal antibody (Sigma-Aldrich). They were then treated with 1.0 ng/ml BMP4 for 48 h.

Immunocytochemistry

Neurospheres were cultured for 2 days in 24-well plates on coverslips coated with 15 μg/ml poly-L-ornithine (Sigma-Aldrich) and 10 μg/ml fibronectin (Gibco, Carlsbad, CA, USA). Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature (RT) and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Neurospheres were then washed with PBS, incubated for 30 min at RT with blocking solution (10% normal goat serum in PBS), and incubated overnight at 4 °C with blocking solution containing anti-Nestin (BD bioscience, Franklin Lakes, NJ, USA), anti-neuron-specific class iii beta-tubulin (Tuj1) (Covance, Princeton, NJ, USA), anti-glial fibrillary acidic protein (GFAP; Biogenex, San Ramon, CA, USA), or anti-myelin basic protein (MBP; Abcam, Cambridge, MA, USA). The neurospheres were washed with PBS, incubated for 1 h at RT with the Alexa Fluor 488- or Alexa Fluor 555-conjugated IgG secondary antibody (Molecular Probes, Eugene, OR, USA), washed with PBS again, counterstained for 5 min with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), and mounted in Fluorescence Mounting Medium (DAKO, Carpinteria, CA, USA).

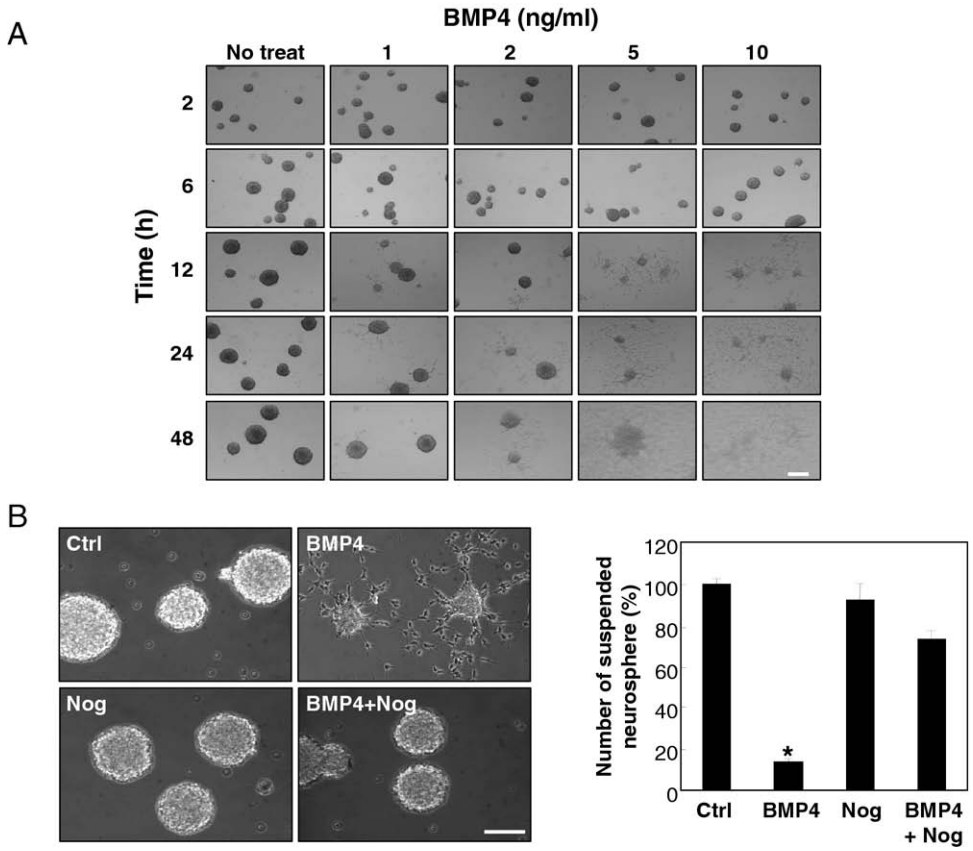

Fig. 1. Effect of BMP4 on attachment of neurospheres. (A) NSCs were isolated from E14.5 rat embryos and grown in N2 medium containing 10 ng/ml bFGF and 20 ng/ml human EGF to form neurospheres. The neurospheres were treated with 1.0 ng/ml BMP4 for 48 h. Images of neurospheres were taken at varying times after treatment with 1.0–10 ng/ml of BMP4. The scale bar represents 200 μm. (B) Images showing the morphologies of neurosphere NSCs at 48 h after treatment of BMP4 (left). A subset of cells was pre-treated 100 ng/ml of Noggin for 1 h and then BMP4 was treated. The scale bar represents 200 μm. The numbers of floating neurospheres were counted from the images, and relative percentages of floating neurospheres were determined (right). Error bars indicate the standard deviation of three independent experiments. * $P<0.05$ by ANOVA and Scheffe's test.

Immunoblot analysis

Neurospheres were incubated at 4 °C for 10 min in chilled RIPA buffer [25 mM Tris, pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS)] supplemented with 1 mM Na-Fluoride, 1 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich). Neurospheres were then centrifuged at 15,000×*g* for 30 min at 4 °C. The resulting protein lysate was boiled at 100 °C, separated on a 10–12% SDS polyacrylamide gel, and subjected to immunoblot analysis. Protein lysates were probed with antibody to N-cadherin (BD Biosciences), phosphorylated Akt (p-Akt; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (Abcam) and then with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Calbiochem, La Jolla, CA, USA). Protein bands were visualized using enhanced chemiluminescence and a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

RT-PCR

Neurospheres were harvested, and total RNA was isolated using Trizol (Invitrogen). Two micrograms of the total RNA was reverse transcribed using 200 U of reverse transcriptase in a 20 μl reaction at 42 °C for 60 min. The resulting cDNA (2 μl) was amplified in a 20 μl reaction containing 10 mM dNTP, 10 pmol of the primer set, and 1 U of Taq DNA polymerase. The following primer sets were used: N-cadherin, forward 5′-caagagcttgtcagaatcagg-3′ and reverse 5′-catttggatcatccgcatc-3′; NCAM, forward 5′-caaaaatgacgaagccgaat-3′ and reverse 5′-gtggacgttctccaggtgat-3′; GFAP, forward 5′-acctgcgaccttgagtcctt-3′ and reverse 5′-tacaggaatggtgatgcggt-3′; and Hprt, forward 5′-cctgctggattacattaaagcgct-3′ and reverse 5′-gtcaagggcatatccaacaacaaa-3′; Tuj1, forward 5′-cagcaaagtgcgtgaggagt-3′ and reverse 5′-gcggaagcagatgtcgtaga-3′.

Flow cytometry

Neurospheres were harvested, dissociated with trypsin-EDTA for 10 min at 37 °C, and washed with PBS. Cells were fixed with 4% paraformaldehyde, washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 15 min on ice. Cells were stained with anti-GFAP and anti-PCNA (Santa Cruz Biotechnology) in PBS on ice. One h later, cells were washed with PBS, incubated for 30 min in appropriate secondary antibody in PBS, and analyzed using the FACScan fluorescence activated cell sorting (FACS analyzer Beckman, Europark, Krefeld, Germany).

Time-lapse video microscopy

Neurospheres grown on poly-L-ornithine and fibronectin-coated 8-well chambers were treated with rhBMP4 (R&D Systems, Minneapolis, MN, USA), along with LY294002, Noggin, or anti N-cadherin. Images were recorded using time-lapse video microscopy (Nikon, Melville, NY, USA) for 24 h in a humidified, 5% CO_2 atmosphere at 37 °C. Bright field images were captured every 20 min and reconstructed into movies using NIS-Elements AR 3.00

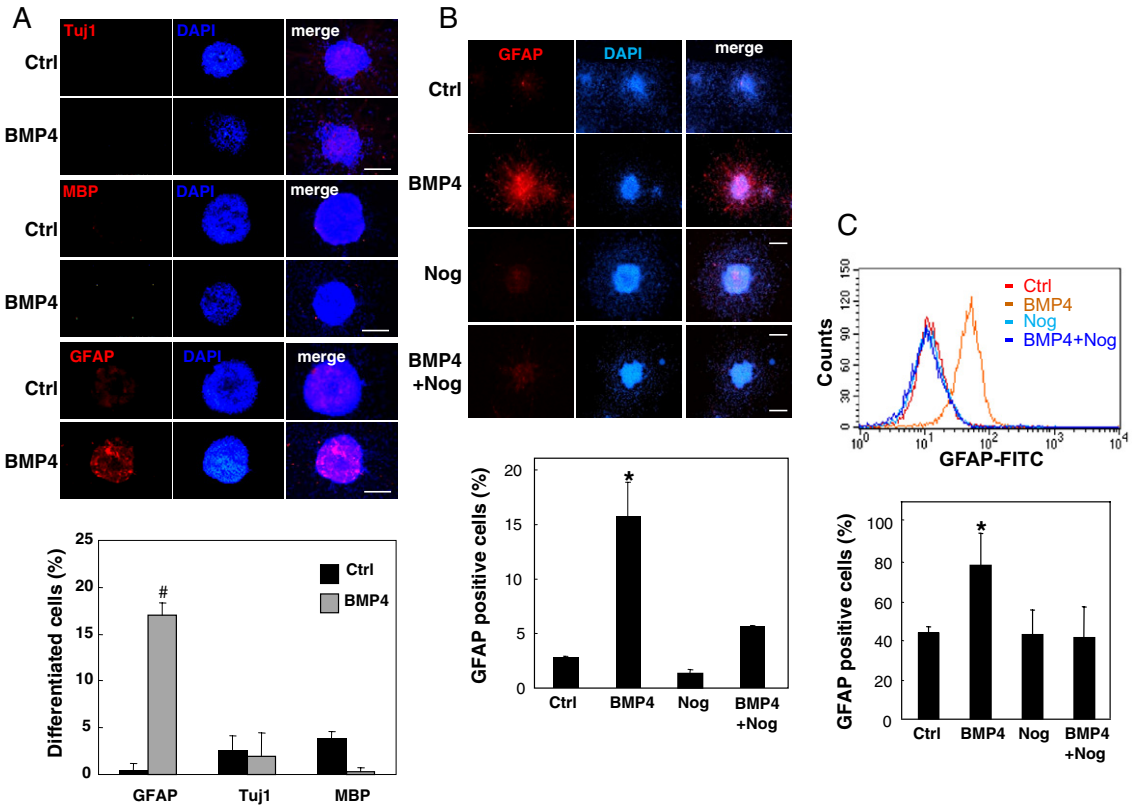

Fig. 2. Effect of BMP4 on the differentiation of neurosphere NSCs. Neurospheres were plated onto coverslips, exposed to BMP4 (1.0 ng/ml) with or without a 1-h pre-treatment with Noggin (100 ng/ml), and cultured for 2 d. (A, B) Immunocytochemical analysis of neurosphere NSCs using GFAP, Tuj1, and MBP antibody (left). Nuclei were counterstained with DAPI (blue). The relative percentages of GFAP-, Tuj1-, or MBP-positive, differentiated cells were estimated from the immunocytochemical data (right). In the (A) panel scale bars represent 100 μm and (B) panel scale bars represent 200 μm. Error bars indicate the standard deviation of three independent experiments. (C) FACS analysis of GFAP expression in neurosphere NSCs. Quantification of GFP-positive cells is shown in the lower panel. * $P<0.05$ by ANOVA and Scheffe's test and # $P<0.05$ by *t*-test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

(Nikon). The migration distance of each of the neurosphere cells were estimated by calculating distance from the center of the neurosphere to the edge of migration at the single cell level. At least 10 cells used for statistical analysis for estimation of the relative migration distance of neurosphere NSCs.

Statistical analysis

Data are expressed as mean±standard error of the mean (SEM). Data analyses were performed by one-way ANOVA followed by the Scheffe's post hoc test between groups using PASW statistics 17.0 software (SPSS Inc., Chicago, IL, USA) or *t*-test. $P<0.05$ was considered significant for three independent experiments.

RESULTS

BMP4 induces attachment of neurospheres and astrocytic differentiation of neurosphere NSCs

NSCs isolated from the subventricular zone of E14.5 Sprague–Dawley rats formed neurospheres when grown in medium containing bFGF and EGF, as previously reported (Deleyrolle and Reynolds, 2009). Stemness of the NSCs was confirmed by immunocytochemical staining of neurospheres with the NSC marker Nestin. The neurosphere NSCs retained the stem cell characteristic, therefore, showed Nestin positive signal for most cases, and that signal totally disappeared in the neurosphere; NSCs differentiated into astrocytes (monitored by GFAP staining) by BMP4 treatment (data not shown). We found that BMP4 treatment led to the dissociation of the neurospheres and attachment of neurospheres to the surface of the plates (Fig. 1A). The adherent characteristic became noticeable after 12 h incubation in the presence of BMP4 concentrations greater than 1.0 ng/ml (Fig. 1A). BMP4-induced attachment of the neurospheres was blocked by treatment of the cells with Noggin, a BMP4 antagonist (Fig. 1B; left panel). Accordingly, BMP4 decreased the number of floating neurospheres (14.3%) due to increased neurosphere adherence, and co-treatment with Noggin partially restored this number (73.0%) (Fig. 1B; right panel). The attachment of neurospheres by BMP4 occurs regardless of the presence of bFGF (data not shown), indicating that the attachment of neurosphere is attributed by BMP4 signaling.

To determine whether BMP4 alters the differentiation potential of adherent neurosphere NSCs, we monitored the expression of differentiation markers by immunocytochemical analyses. We found that BMP4 did not appreciably affect the differentiation of NSCs into neurons or oligodendrocytes, as assessed using antibodies to Tuj1 or MBP, respectively (Fig. 2A). However, immunocytochemical staining for GFAP revealed that BMP4 significantly increased the astrocytic differentiation of neurosphere NSCs

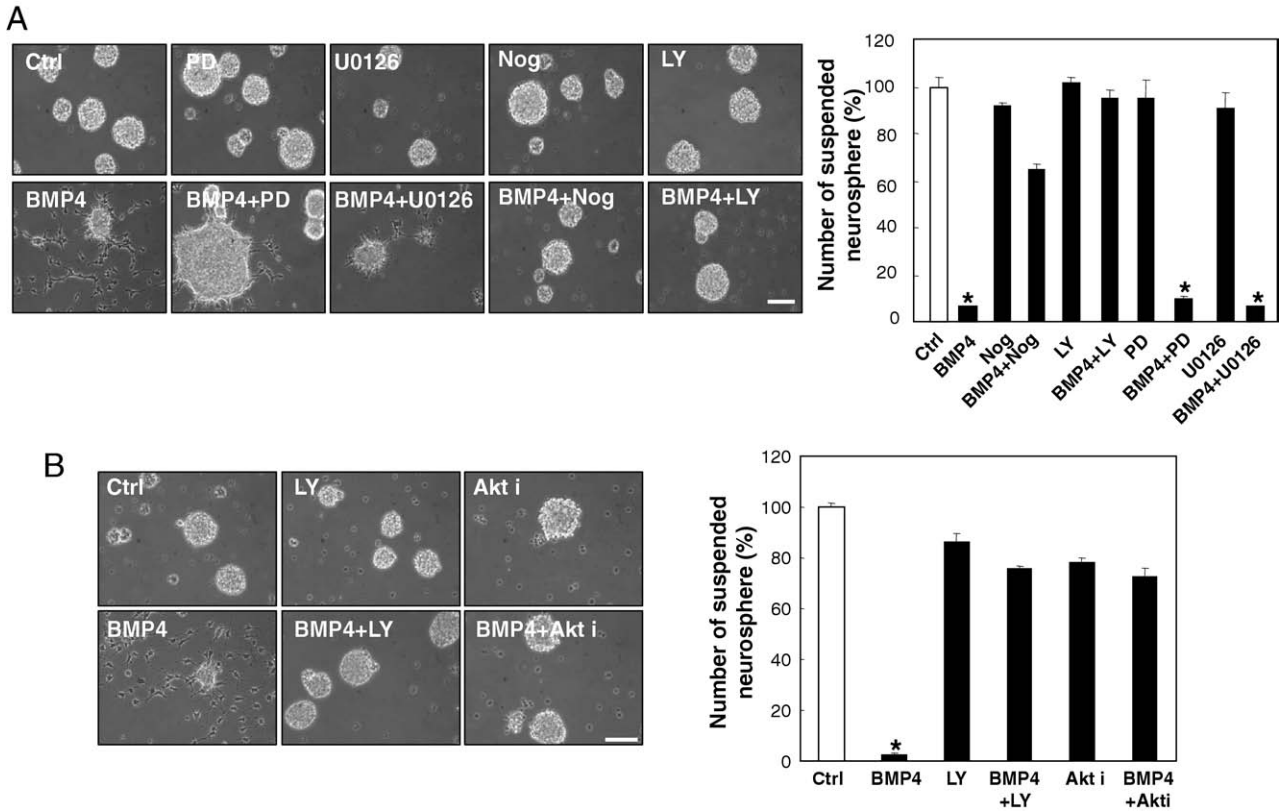

Fig. 3. Effect of inhibitors of the PI3 kinase-Akt or ERK pathway on BMP4-induced adhesion of the neurospheres. (A, B) The neurospheres were grown as described in Fig. 1. A subset of cells received 10 μM LY294002, 10 μM PD98059, 5 μM U0126, or 25 nM of Akt inhibitor for 1 h before BMP4 treatment. Phase contrast images were taken after 2 d in culture (left). The scale bar represents 200 μm. The relative numbers of the floating neurospheres were estimated from phase contrast images (right). Error bars indicate the standard deviation of three independent experiments. * $P<0.05$ by ANOVA and Scheffe's test.

(Fig. 2A, B; the quantitative data for differentiated cells are shown in the lower panels). The BMP4 effect on the specific astrocyte differentiation was further confirmed by specific increment of mRNA level of GFAP but not that of Tuj1 at 2 and 3 days after treatment of BMP4 (data not shown). The role BMP4 on specific astrocytic differentiation of neurosphere NSCs were further confirmed by blockade of the BMP4-mediated GFAP induction by co-treatment of Noggin (Fig. 2B). To further characterize the BMP4 effects on the neurosphres, FACS analysis was performed with the attached cells by BMP4. The GFAP-positive cells were similarly increased by BMP4 treatment, and that effect was blocked by co-treatment of Noggin (Fig. 2C; quantitative data are shown in lower panel). Together, these findings show that BMP4 induces the attachment of neurospheres and astrocytic differentiation of neurosphere NSCs.

The PI3 kinase/Akt pathway mediates BMP4-induced attachment of neurospheres

BMP4 activates the PI3 kinase-Akt and extracellular signal-regulated kinase (ERK) pathways in retinal stem cells (Haynes et al., 2007), but whether it activates these pathways in attached neurospheres is unknown. To understand whether the PI3 kinase-Akt and ERK pathways participate in BMP4-induced attachment of neurospheres, we analyzed neurosphere attachment in the presence of specific inhibitors for these pathways. BMP4-induced attachment of neurospheres was not affected by the MEK inhibitors, PD98059 or U0126 (Fig. 3A). However, it was critically abolished by the PI3 kinase inhibitor, LY294002, as seen by re-formation floating neurospheres (Fig. 3A, B; quantification of the relative numbers of floating neurospheres are shown in right panel). BMP4-induced attachment and dissociation of neurospheres were also blocked by a specific Akt inhibitor (Fig. 3B), confirming that the PI3-Akt pathway mediates these effects of BMP4. Finally, we also investigated whether the PI3 kinase pathway participates in BMP4-induced astrocytic differentiation. Indeed, we found that BMP4-induced astrocytic differentiation of neurosphere NSCs was abolished by the PI3 kinase inhibitor LY294002, as shown by both immunocytochemical and FACS analyses (Fig. 4A, B; quantitative data are shown in right panels).

BMP4 stimulates attachment of neurospheres and astrocytic differentiation of neurosphere NSCs via PI3 kinase pathway-mediated upregulation of N-cadherin

N-cadherin is a key factor mediating cell adherence. Transcription of N-cadherin is regulated by Sp1 and Ap1 (Derycke and Bracke, 2004), which are known to be activated by the ERK and PI3 kinase pathways (De Wever et

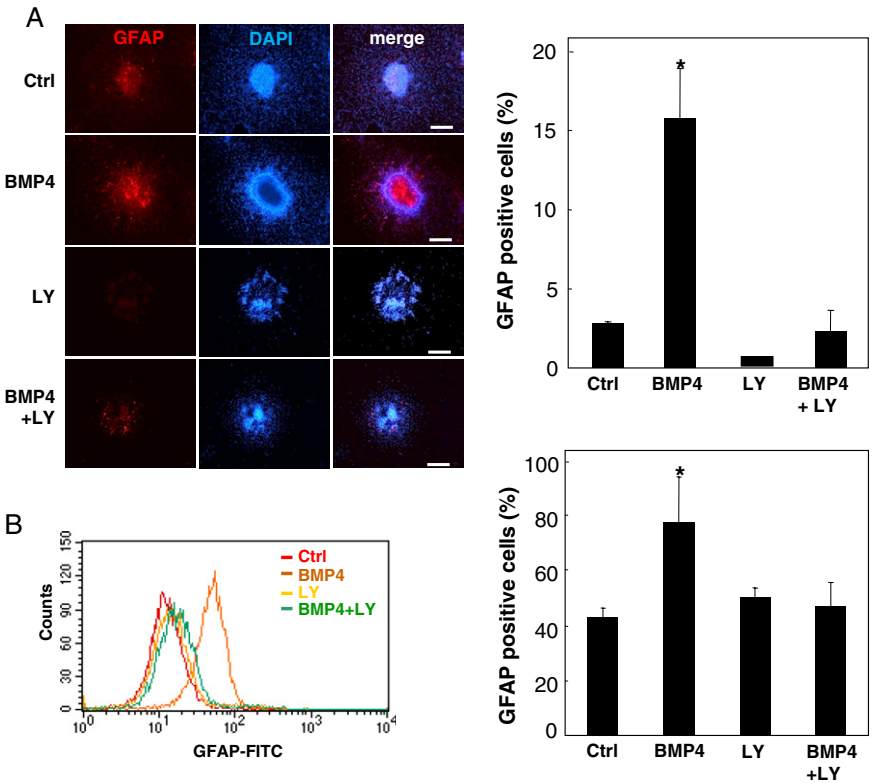

Fig. 4. Effect of a PI3 kinase inhibitor on BMP4-induced differentiation of neurosphere NSCs into astrocytes. Neurospheres were grown and treated with BMP4 and/or LY294002 as described in Fig. 3A. (A) Immunocytochemical analysis of neurosphere NSCs using GFAP antibody, as described in Fig. 3A. Nuclei were counterstained with DAPI. The scale bar represents 200 μm. (B) FACS analysis of GFAP-positive cells. The relative percentages of GFAP-positive cells are shown to the right. In (A) and (B), error bars indicate the standard deviation of three independent experiments. * $P<0.05$ by ANOVA and Scheffe's test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

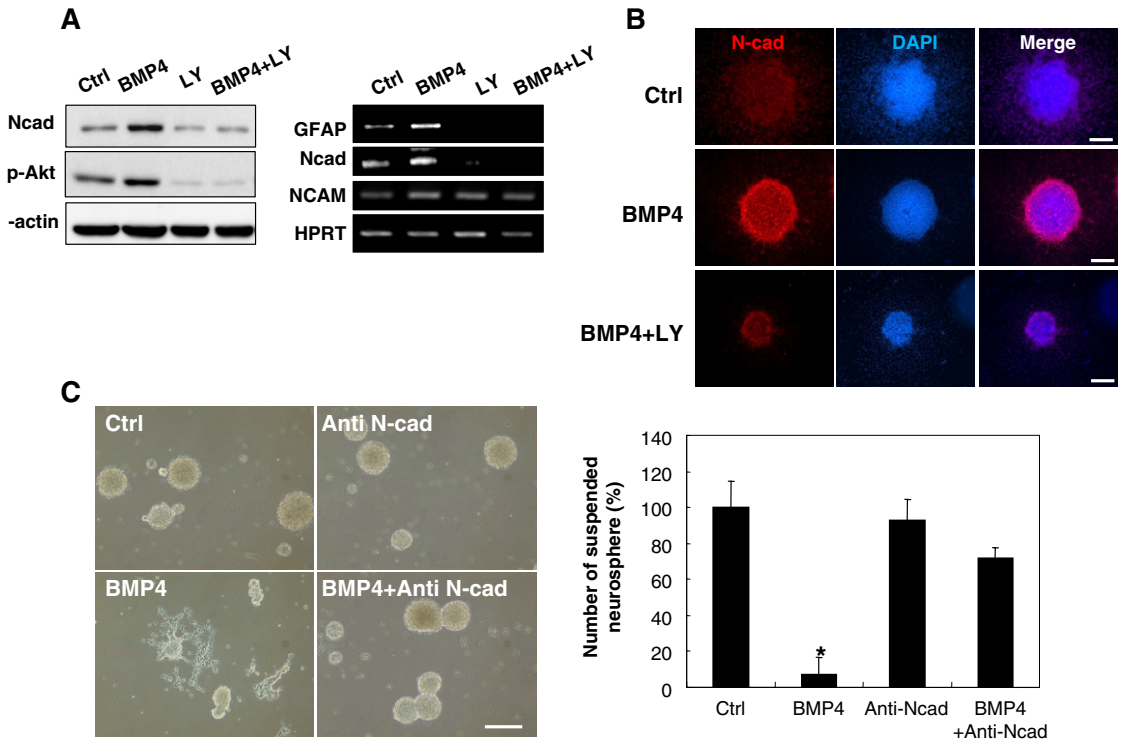

Fig. 5. N-cadherin is induced via the PI3 kinase-Akt pathway and participates in attachment of neurosphere NSCs. Neurospheres were grown and treated with BMP4 and/or LY294002 as described in Fig. 3A. (A) Whole-cell extracts were subjected to immunoblotting using antibody to N-cadherin, p-Akt, and β-actin (left). RT-PCR was performed using total RNA to analyze mRNA levels of N-cadherin, NCAM, GFAP, and HPRT (right). (B) Immunocytochemical analysis of N-cadherin. Nuclei were counterstained with DAPI (blue). (C) Effect of N-cadherin antibody on BMP4-induced attachment of neurosphere NSCs. Neurospheres were grown and treated with 1.0 ng/ml BMP4 and/or 10 μg/ml N-cadherin monoclonal antibody as described in Fig. 3A. Representative phase contrast images are shown to the left, and the relative percentages of suspended neurospheres are shown to the right. The scale bar represents 200 μm. * $P<0.05$ by ANOVA and Scheffe's test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

al., 2004; Rieger-Christ et al., 2004). Therefore, we investigated the effect of BMP4 on N-cadherin expression and the possible involvement of the PI3 kinase-Akt pathway in mediating BMP4-induced changes in N-cadherin expression. Immunoblot analysis revealed that the levels of both N-cadherin and activated p-Akt increased by BMP4 treatment (Fig. 5A). Interestingly, BMP4-stimulated induction of N-cadherin and activation of Akt were completely blocked by LY294002 (Fig. 5A; left panel). The ability of LY294002 to block BMP4-induced N-cadherin induction was also apparent by immunocytochemical staining of the neurosphere NSCs (Fig. 5B). As with protein levels, the mRNA levels of both GFAP and N-cadherin were increased by BMP4 through the PI3 kinase-Akt pathway (Fig. 5A; right panel). These findings show that BMP4 induces attachment and astrocytic differentiation of neurosphere NSCs by activating the PI3 kinase pathway and inducing N-cadherin expression.

To further investigate the role of BMP4-induced upregulation of N-cadherin in the adherence of neurosphere NSCs, we analyzed neurosphere adherence in the presence of a neutralizing N-cadherin monoclonal antibody. As shown in Fig. 5C, this N-cadherin antibody markedly inhibited the reduction in floating neurospheres and attachment of neurosphere NSCs resulting from BMP4 treatment. Thus, BMP4 induces attachment of neurospheres, at least partly, through induction and secretion of N-cadherin.

BMP4 stimulates migration of neurosphere NSCs via PI3 kinase-Akt pathway-mediated upregulation of N-cadherin

Finally, we tested whether BMP4-induced attachment of neurosphere is associated with the migration of neurosphere NSCs. Migration of neurosphere NSCs after BMP4 treatment was monitored using time-lapse microscopy. We found that migration was greater in neurosphere NSCs treated with BMP4 for 24 h than in non-treated control cells (Fig. 6; quantitative data are shown in the right panel). We also found that BMP4-induced migration of neurosphere NSCs was blocked by Noggin, LY294002, or N-cadherin monoclonal antibody.

DISCUSSION

The neurosphere culture system has been an important tool for expanding NSCs *in vitro* and has provided a source of cells for transplantation and treatment of CNS disorders (Reynolds et al., 1992; Eriksson et al., 2003). Although we did not confirm homogeneity, nerosphere NSCs showed strong positive signal for Nestin, the neural stem cell

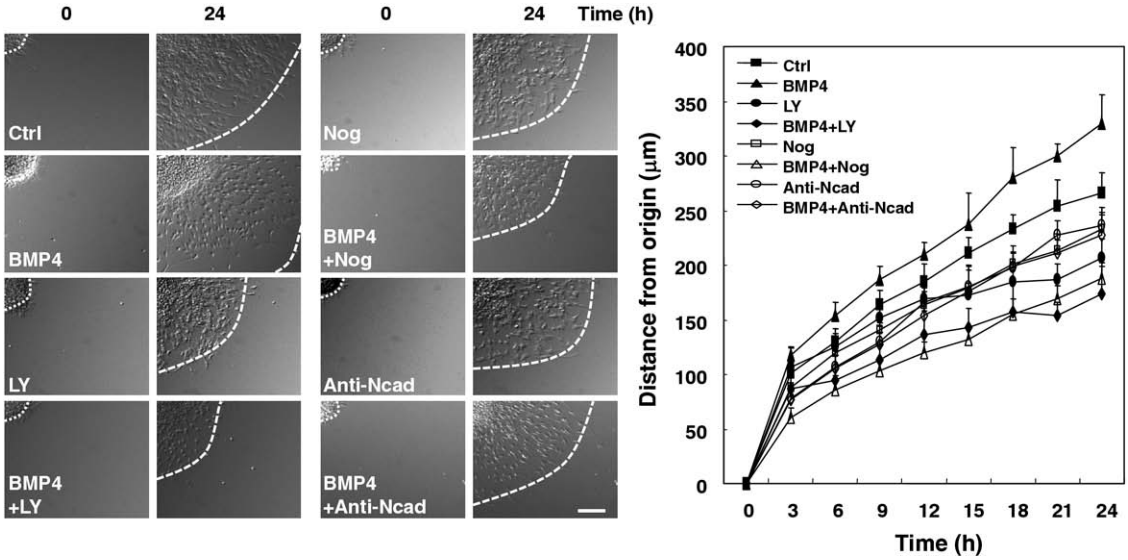

Fig. 6. Effect of a PI3 kinase inhibitor on BMP4-induced migration of neurosphere NSCs. Neurospheres were seeded on 8-well chamber plates coated with 15 μg/ml poly-L-ornithine and 10 μg/ml fibronectin. They were then pre-treated with 10 μM LY294002, 100 ng/ml of Noggin, or 10 μg/ml monoclonal anti N-cadherin and exposed to 1.0 ng/ml BMP4 for varying times up to 24 h. Migration of cells from the center of neurospheres was observed by time-lapse imaging microscopy (left). The white dotted lines and dotted arrows indicate the edge of the migrating cells and the direction from the migration origin, respectively. The scale bar represents 100 μm. The relative migrating distances from the center are shown to the right. Error bars indicate the standard deviation of three independent experiments.

marker (Data not shown). It is known that NSCs in neurospheres retain basic stem cell characteristics such as self-renewal and multipotency (Reynolds and Rietze, 2005). However, the physiological significance of attachment of neurosphere has been unclear.

In this study, we show that BMP4 induces selective astrocytic differentiation of neurosphere NSCs that is accompanied by the attachment of neurospheres and migration of neurosphere NSCs. These cellular behaviors are acquired by overcoming the effect of EGF/bFGF in stimulating neurosphere formation. BMP4 stimulates attachment of neurospheres via the PI3K/Akt pathway and that occur independently of bFGF. The ability of BMP4 to induce neurosphere attachment, astrocytic differentiation, and neurosphere NSC migration was primarily due to its activation of the PI3 kinase-Akt pathway, as seen by the blockade of these effects by inhibitors of PI3 kinase and Akt. The PI3 kinase-Akt pathway may have a specific role in these BMP4-activated processes, as ERK pathway inhibitors had no effect on these phenomena.

Importantly, BMP4 not only activated the PI3 kinase-Akt pathway, but also induced expression of N-cadherin, a cell adherence factor that mediates cell-cell contact and neurite outgrowth (Utton et al., 2001). The ability of LY294002 to inhibit BMP4-induced N-cadherin upregulation and Akt activation indicates that the PI3 kinase-Akt pathway participates in N-cadherin induction by BMP4. This result is consistent with the finding that the PI3 kinase pathway up-regulates N-cadherin expression in bladder cells (Rieger-Christ et al., 2004). We found that BMP4 induces N-cadherin expression and astrocytic differentiation by altering transcription, as seen by increased mRNA levels of N-cadherin and GFAP. In addition, experiments with N-cadherin antibody clearly showed that N-cadherin mediates the attachment of nerospheres and migration of neurosphere NSCs elicited by BMP4. N-cadherin is abundantly expressed in the nervous system, especially in astrocytes (Tran et al., 2008). This, taken with the fact that N-cadherin participates in cell migration associated with NSC differentiation and nervous system development (Derycke and Bracke, 2004), supports the idea that the BMP4-induced N-cadherin up-regulation is important for the astrocytic differentiation and migration of NSCs in neurospheres.

The adherence of neurospheres and astrocytic differentiation of neurosphere NSCs were highly sensitive to BMP4 concentration. Indeed, these effects were observed at concentrations as low as 1.0 ng/ml. Moreover, BMP4 selectively induced differentiation of neurosphere NSCs into astrocytes, with no significant differentiation into neuronal cells or oligodendrocytes being observed. Previous studies have uncovered a relationship between migration and differentiation of NSCs (Liu et al., 2009 and Mani et al., 2010). Here, we have extended these findings by showing that a significant stimulation of neurosphere adherence (elicited by BMP4) is related to the migration of NSCs as well as the differentiation of these cells into astrocytes. These novel findings suggest that this system could be used as a transplantation therapy to treat CNS diseases related to defects in astrocytes, such as Parkinson's disease (Teismann and Schulz, 2004) and Alexander disease (Hagemann et al., 2009).

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