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Bone Morphogenetic Proteins Inhibit Ciliogenesis of Ependymal Cells *in Vitro*

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Ependymal cells have an essential role in regulating the dynamics of the cerebrospinal fluid flow by the movement of their multiple cilia. Impaired generation or function of cilia could cause hydrocephalus due to the disordered dynamics of the cerebrospinal fluid flow. However, molecular bases regulating differentiation of the ependymal cells and their ciliogenesis have not been fully elucidated. We report here that bone morphogenetic proteins (BMPs), growth factors orchestrating tissue architecture throughout the body, inhibit ciliogenesis during ependymal cell differentiation in primary cell culture. Previous *in vitro* study has reported that ectopic expression of Smad6 and Smad7 promotes differentiation of embryonic stem cells into multi-ciliated ependymal-like cells. Since Smad6 and Smad7 have been known as the intracellular inhibitory factors of the BMP signaling pathway, the activation of the pathway could cause a deficit in ciliogenesis of ependymal cells. To examine whether activation of the pathway affects ciliogenesis, we investigated the effects of two BMPs, BMP2 and BMP4, on the ependymal differentiation of the primary cultured cells prepared from the neonatal mouse brain. Supplementation of BMP2 or BMP4 in culture media significantly reduced the number of cells with multiple cilia among the total cells, while most of the cells expressed FoxJ1, a master regulator of ciliogenesis. Activation of the pathway was confirmed by the phosphorylation of intracellular Smad1/5/8, downstream factors of the BMP receptors. These *in vitro* results suggest that inhibition of the BMP signaling pathway might be essential for ciliogenesis during the ependymal cell differentiation *in vivo*.

Keywords: bone morphogenetic proteins; ciliogenesis; differentiation; ependymal cells; primary cell culture
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Introduction

Ependymal cells are classified in a type of glial cells, which cover the surface of ventricles and regulates the transfer of various molecules such as water, glucose, and ions between the brain and cerebrospinal fluid (CSF) in the brain (Del Bigio 2010). The ependymal cells do not only form a "brain-CSF barrier" but also generate CSF flow by a movement of their multiple cilia that project from an apical cell surface to ventricular lumens (Del Bigio 2010). An impaired generation or function of ependymal cells could cause congenital hydrocephalus (Lee 2013); therefore, it is essential to elucidate the molecular mechanism of ependymal cell differentiation for further understanding of hydrocephalus development.

As with neurons, astrocytes, and oligodendrocytes, the ependymal cells are differentiated from radial glia in brain development during embryogenesis. Progenitors of the ependymal cells are generated during embryonic days 14 to 16 in mice and then mature to multi-ciliated cells during the first postnatal week (Spassky et al. 2005). Recently, several factors that regulate the differentiation of the ependymal cells have been reported (Kyrousi et al. 2017). For example, transcription factors, forkhead box protein J1 (FoxJ1)

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and regulatory factor X3 (RFX3) have been reported to play a critical role in the differentiation of radial glia into ependymal cells (Choksi et al. 2014). The differentiation of ependymal cells also requires the other factors, such as the homeobox protein Six3, which maintains anterior forebrain identity during early development, (Lavado and Oliver 2011), Mcidas, which is mutated in human mucociliary clearance disorder, and GemC1/Lynkeas (Kyrousi et al. 2015), and p73, a homolog of tumor suppressor p53 (Gonzalez-Cano et al. 2016). Although there is no doubt that the intrinsic developmental program by these factors is essential for the differentiation of ependymal cells, environmental or extrinsic factors regulating differentiation of the ependymal cells have not been fully elucidated.

Bone morphogenetic proteins (BMPs) are growth factors originally discovered by their ability to induce the formation of bone and cartilage (Bandyopadhyay et al. 2013). BMPs are now considered to constitute a group of pivotal morphogenetic signals, orchestrating tissue architecture throughout the body including the nervous system (Liu and Niswander 2005). BMPs elicit their cellular effects through specific receptors and the signal is propagated from the cell membrane to the nucleus via intracellular Smad proteins, Smad1/5/8. It is also known that Smad6 and Smad7 are the intracellular inhibitory factors of the BMP signaling pathway. A previous *in vitro* study using tetracycline-regulated Smad6- and Smad7-expressing embryonic stem cells has shown that ectopic expression of Smad6 and Smad7 promotes differentiation of stem cells into ependymal-like cells with multiple cilia (Nishimura et al. 2010). In addition, BMP receptors were expressed in the ependymal stem cells and regulated production of astrocytes on astrogliosis after spinal cord injury (North et al. 2015). These studies suggest that the activity of the BMP signaling pathway could regulate the differentiation of ependymal cells.

We hypothesized that activation of the BMP signaling pathway affected ependymal cell differentiation. We first checked the expression of BMPs in ALLEN Developing Mouse Brain Atlas (https://developingmouse.brain-map.org/), and the atlas suggested that progenitor cells might be exposed to BMPs during ependymal differentiation. We investigated here the effects of two BMPs, BMP2 and BMP4, on ependymal differentiation using primary brain cell culture. Supplementation of BMP2 or BMP4 in media caused a significant decrease in the number of cells with multiple cilia, while FoxJ1, a master regulator of ciliogenesis, was expressed in most of the cells in the presence of BMP2 or BMP4. The activation of the BMP signaling pathway was confirmed by the phosphorylation of Smad1/5/8 protein. The results suggested that BMPs inhibit ciliogenesis during the ependymal cell differentiation, but do not affect cell fate. Our *in vitro* results suggest that inhibition of the BMP signaling pathway might be essential for ciliogenesis during the ependymal cell differentiation *in vivo*.

Materials and Methods

Animals

C57BL/6J mice were purchased from Japan SLC and maintained in the animal facility of Tohoku University School of Medicine. All experiments were performed following the National Institute of Health guidelines for the care and use of laboratory animals and approved by the Committee for Animal Experiments in Tohoku University (2013CrA-005).

Primary ependymal cell culture

Primary ependymal cell culture was performed as described previously (Delgehyr et al. 2015). Initially, the telencephala were dissected from pups on postnatal days 0 to 2. The dissected telencephala were soaked in enzymatic digestion solution (30 μL of Papain, 15 μL of 1% DNase I, 24 μL of 12 mg/mL $_{L}$-Cysteine in 1 mL of Dulbecco's modified Eagle's medium (DMEM)/Glutamax with 1% Penicillin/Streptomycin per telencephalon) to dissociate cells. The dissociated cells were inoculated in the flasks coated with Poly-$_{L}$-Lysine containing DMEM supplemented with 10% fetal bovine serum. The cells were incubated for 7 days until they reached confluence. When the cells reached confluence, the flasks were tightly closed and shook overnight to remove weakly attached cells, such as differentiated oligodendrocytes and neurons. Progenitor cells attached to flasks after shaking were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) treatment. Detached cells were inoculated on coverslips coated with Poly-$_{L}$-Lysine at a cell density of 2×10^5 cells per 20 μL. The cells were subsequently incubated in DMEM with 10% fetal bovine serum for one day, followed by incubation in DMEM without serum for designated days, and used for immunocytochemistry.

In the previous paper (Delgehyr et al. 2015), it is described that the cells differentiate and grow multiple cilia during the incubation without fetal bovine serum, and 70-80% cells reach ependymal cells within 15 days. However, it was not investigated how many days are needed to reach the plateau of ependymal cell differentiation. To confirm whether ependymal differentiation progress similarly as the previous paper, and to investigate how many days are needed to reach the plateau of ependymal cell differentiation, we initially cultured cells for 0, 3, 7, 14, and 21 days in DMEM without fetal bovine serum, and examined the number of cells with multiple cilia by the use of immunocytochemistry in the first experiment of our study.

In the second experiment, recombinant human/mouse/rat BMP2 protein (R&D systems, 355-BM-010) or recombinant mouse BMP4 protein (R&D systems, 5020-BP-010) was added in DMEM without fetal bovine serum to evaluate the effect of BMP proteins on the differentiation of ependymal cells. Among various BMPs, we chose BMP2 and BMP4 because these BMPs were used in previous stud-

ies which investigated the role of BMPs in the differentiation of brain cells such as astroglia (Gross et al. 1996; Mabie et al. 1997) and neuronal cells (Li et al. 1998). The solvent used for dissolving BMP2 and BMP4 was 4 mM HCl with 0.1% bovine serum albumin, which is recommended in the product datasheet of the maker. Stock solutions were dispensed into small aliquots and stored at –20°C. The dispensed stock solutions were used up for each experiment. One μl of BMP2 or BMP4 stock solution (5, 10, and 100 μg/mL) was added to 1 mL of DMEM, so that final concentrations of BMPs were 5 ng/mL, 50 ng/mL, and 100 ng/mL. Under control condition, 1 μL of the vehicle (4 mM HCl with 0.1% bovine serum albumin) was added to 1 mL of DMEM. Cells were cultured for 2 weeks in the medium with or without BMPs and underwent immunocytochemistry.

Immunocytochemistry and cell counting

Cells were fixed in 4% paraformaldehyde for 6 min at room temperature. The cells were then processed for immunofluorescence using mouse antibody against β-Tubulin IV (1:200, Sigma-Aldrich, T7941) to stain cilia. Rabbit anti-FoxJ1 polyclonal antibody (1:200, Sigma, HPA005714), mouse anti-nestin monoclonal antibody (1:400, BD, BD611658), and rat anti-BrdU monoclonal antibody (1:200, AbD serotec, OBT0030) were also used as primary antibodies. Donkey anti-mouse IgG-Alexa488 (1:500, Jackson ImmunoResearch, 715-545-150), donkey anti-rabbit IgG-Cy3 (1:500, Jackson ImmunoResearch, 712-165-153), and donkey anti-rat IgG-Cy3 (1:500, Jackson ImmunoResearch, 711-165-152) were used as secondary antibodies. Nuclei were counterstained with DAPI. Fluorescent images of cells were obtained using a fluorescent microscope (BZ-X710, Keyence). The total number of cells was determined by counting DAPI stained nuclear signals using Fiji (ImageJ) (Schindelin et al. 2012). The number of FoxJ1 positive cells was also determined using Fiji. Numbers of BrdU or nestin-positive cells or multi-ciliated cells were counted manually.

BrdU labeling

Three days after changing from culture medium with a fetal bovine serum to medium without serum, 5-Bromo-2'-deoxy-uridine (BrdU) (Roche, Mannheim, Germany) was supplemented into the culture at 10 μM (final concentration). Cells were cultured for 24 hours at 37°C in a CO_2 incubator, rinsed with PBS once, and fixed with 4% PFA for 15 min at room temperature. Cells were treated with 2 N HCl for 30 min at room temperature to hydrolyze DNA and used for immunostaining with antibodies against BrdU and nestin.

Immunoblotting

The primary cell culture for immunoblotting was prepared as described above. 10^6 cells of purified progenitor cells were seeded in a well of 12-well plate coated with Poly-L-Lysine and cultured for a day with a medium containing 10% FBS. After induction of differentiation by replacing media to those without FBS, cells were cultured for 2 days in the presence or absence of BMP2 or BMP4 (50 ng/ml). Control and BMP-treated cells were collected with 100 ml of 1x SDS buffer and denatured at 95°C for 10 min. Electrophoresis and transfer were performed with the DIRECT BLOT system (SHARP, BM-80). Subsequent immunoblotting was performed with iBind™ Flex Western Device (Thermo Fisher Scientific) and iBind™ Flex Fluorescent Detection (FD) Solution Kit (Thermo Fisher ScientificSLF2019). Fluorescent signals were detected with the Odyssey Imaging System (LI-COR). We used primary and secondary antibodies; Rabbit anti-pSmad1/5/8 (1:500, CST, 13820S), Mouse anti-Smad5 (1:100, Santa Cruz, sc-101151), Mouse anti-GAPDH (1:4,000, Abcam, ab8245), IRDye 680RD Goat anti-Rabbit IgG (1:4,000, LI-COR, 926-68071), and IRDye 800CW Goat anti-Mouse IgG (1:3,000, LI-COR, 926-32210).

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance with Tukey post-hoc analysis using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA) or Dunnett's test using JMP14 (SAS Institute Inc., NC). $p < 0.05$ was considered statistically significant.

Results

Initial cell population before induction of differentiation

In the ependymal cell culture, the cells were initially cultured in medium with fetal bovine serum so that progenitor cells proliferate, which is the same process as an initial step of primary astrocyte culture (Sakurai and Osumi 2008), and then cultured in medium without serum to induce differentiation. To investigate the initial cell population before induction of differentiation, we investigated cells using immunostaining just before changing from culture medium with the serum to medium without serum (Fig. 1A). We investigated the expression of FoxJ1, a master regulator of ependymal cell differentiation. Among all the cells, the number of FoxJ1 positive cells was 53.66 ± 3.04% (mean ± standard deviation, n = 3). No cells showed multiple cilia or nestin expression. These results suggest that the initial cell population contains approximately 50% of ependymal progenitor cells.

Chronological progress of ependymal cell differentiation

The previous study (Delgehyr et al. 2015) reported that cells start to differentiate and grow multiple cilia in the media without serum, and 70-80% cells differentiate to multi-ciliated cells within 15 days *in vitro*. In this study, approximately 50% of cells expressed multiple cilia at 14 days after culture in medium without serum (Fig. 1B). To investigate the progress of ependymal cell differentiation, we first cultured cells for different days (0, 3, 7, 14, and 21 days) in media without serum and determined the number

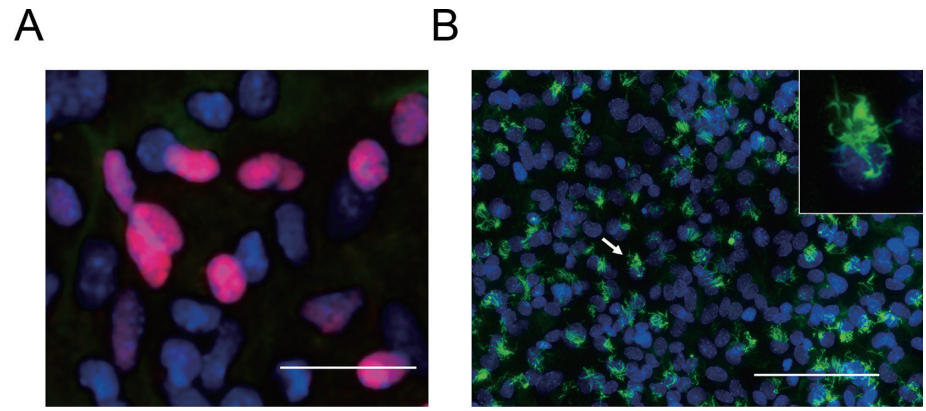

Fig. 1. Cell populations before and after induction of differentiation.
A. Representative images of cell population just before changing from culture medium with a serum to medium without serum to induce differentiation. FoxJ1, a nuclear marker for ciliated cells, is stained in magenta. The nuclei are counterstained with DAPI in blue. β-Tubulin IV antibody is used to image cilia. Even the cells have no multiple cilia, a slight signal from cytosolic β-Tubulin IV is detected as green fluorescence. Scale bar: 20 μm.
B. Representative image of differentiated cells cultured in medium without serum for 14 days. Cilia are imaged in green using a β-Tubulin IV antibody. An inset shows a magnified image of a multi-ciliated cell (arrow). The nuclei are counterstained with DAPI. Scale bar: 100 μm.

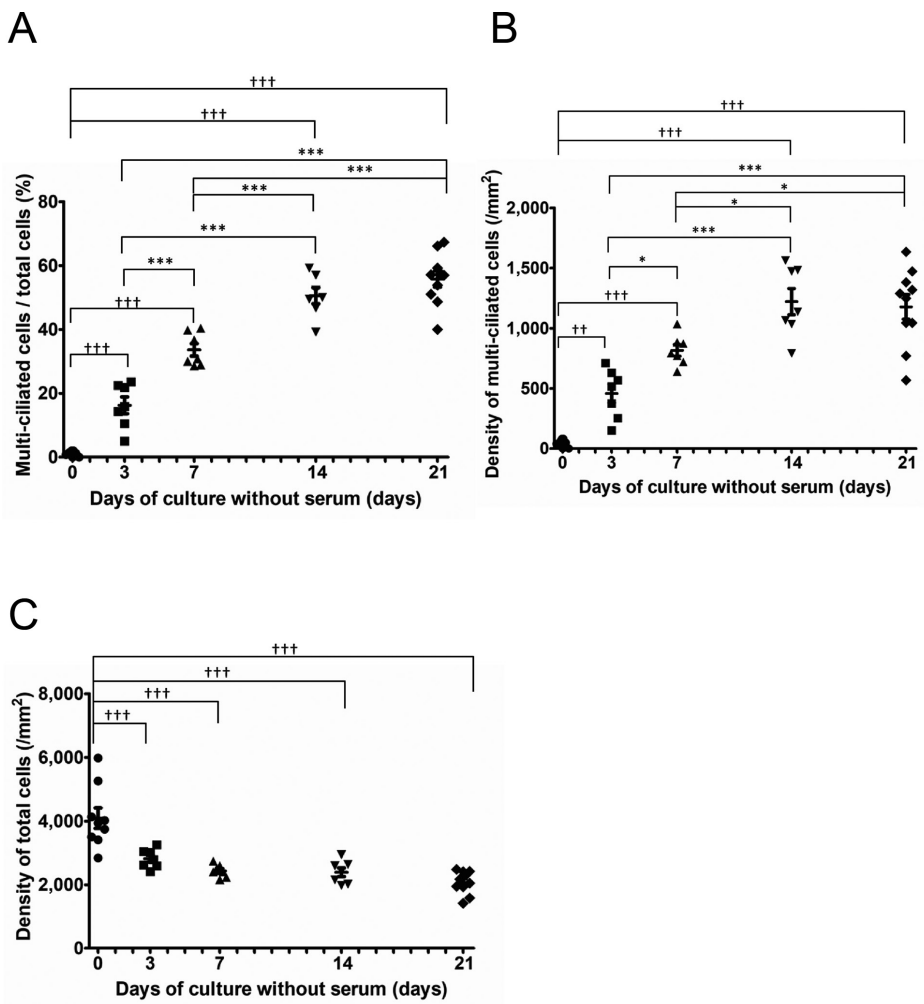

Fig. 2. Chronological progression of cell differentiation.
Temporal changes in the number of multi-ciliated cells per total cells (A), the density of multi-ciliated cells (B), and the density of total cells (C) are shown. n = 7 to 10. Data are mean ± SEM. "†" indicates a significant difference between day 0 and the other days. "*" indicates significant difference between days except day 0.
$^{*}p < 0.05$, **, $^{\dagger\dagger}p < 0.01$, ***, $^{\dagger\dagger\dagger}p < 0.0001$.

of cells with multiple cilia using immunocytochemistry. Multi-ciliated cells appeared from day 3, and the number of multi-ciliated cells showed a significant increase depending on the culture days (Fig. 2A). Significant differences were observed in day 0 vs. 3, day 3 vs. 7, and day 7 vs. 14, but day 14 and 21 of culture showed no significant difference, which suggested that ependymal cell differentiation reached a plateau at 14 days of culture without serum. The density of multi-ciliated cells showed a similar time-course (Fig. 2B). As shown in Fig. 2C, the density of total cells was significantly lower at 3, 7, 14, and 21 days compared to day 0 of culture without serum, which may suggest that some cells had detached or died at the beginning of culture without serum.

Effect of BMPs on ependymal cell differentiation

We then investigated the effect of BMPs on ependymal cell differentiation. BMP2 or BMP4 was added in the culture medium at different concentrations, and the expression of multiple cilia was determined after 14 days of culture in medium without fetal bovine serum. The number of multi-ciliated cells per total cells did not show a significant difference between control and 5 ng/mL BMPs (Fig. 3A). In contrast, the density of multi-ciliated cells showed a significant difference between control and 5ng/mL BMP2 (Fig. 3B). The number of multi-ciliated cells per total cells and density of multi-ciliated cells were significantly decreased in a higher concentration of BMP2 or BMP4 (50 and 100 ng/mL), suggesting that BMP2 and BMP4 suppress ependymal cell differentiation (Figs. 3A, B, and 4). The density of total cells did not show a significant difference among the conditions except between BMP2 at 100 ng/mL and BMP2 at 5 ng/mL (Fig. 3C), which suggests that BMP2 and BMP4 do not affect cell survival, and do not have toxic effects.

It has been reported that BMPs induce phosphorylation of intracellular Smad proteins, Smad1/5/8 (Bandyopadhyay et al. 2013). Bioactivity of BMP2 and BMP4 used in the study was confirmed by phosphorylation of Smad1/5/8 proteins using immunoblotting. Clear signals of phosphorylated-Smad1/5/8 (pSmad1/5/8) were detected in BMP2 or BMP4-treated cells but not in the control cells (Fig. 5A), suggesting that the BMPs were bioactive and their supplementation enhanced the BMP signaling pathway. Since the signal level of non-phosphorylated Smad5 was similar in each condition (Fig. 5B), the clear signals of pSmad1/5/8 were not due to an increase in the protein expression, but to an increase in the protein phosphorylation.

To investigate whether BMP2 and BMP4 affect cell proliferation during ependymal differentiation, we labeled mitotic cells by BrdU at 3 days after changing from culture medium with a fetal bovine serum to medium without serum. Cells were co-stained with nestin, a stem cell marker. In the control condition, 2.48 ± 0.83% (mean ± standard deviation) cells were BrdU positive, 1.18 ± 0.47% cells were nestin-positive, and 0.3 ± 0.23% cells were double-positive among all the cells (Fig. 6). In the presence of BMP2 or BMP4, the number of BrdU positive cells among all the cells was 2.29 ± 0.77% and 2.40 ± 0.58%, which were not statistically different compared to that of the control condition. The number of nestin/BrdU double-positive cells did not show a statistically significant difference in the BMP conditions compared to the control condition. These results suggest that BMP2 and BMP4 could not affect cell proliferation.

We further confirmed cell types using antibodies to FoxJ1 (Fig. 7A), and nestin, a stem cell marker. The number of FoxJ1 positive cells among the total cells did not show a statistically significant difference between the control, BMP2, and BMP4 conditions (88.02 ± 7.70%, 92.26 ± 3.71%, and 91.80 ± 2.51% (mean ± standard deviation) in the control, BMP2, and BMP4 conditions, respectively) (Fig. 7B). However, the number of FoxJ1-positive cells with multiple cilia among the total cells were significantly lower in the BMPs conditions compared to the control condition (37.48 ± 3.61%, 6.78 ± 1.21%, and 5.17 ± 2.86% in control, BMP2, and BMP4 conditions, respectively), while the number of FoxJ1-positive cells without multiple cilia among the total cells were significantly higher in the BMPs conditions compared to the control condition (50.54 ± 8.73%, 85.48 ± 3.91%, and 86.63 ± 5.29% in control, BMP2, and BMP4 conditions, respectively) (Fig. 7B). The number of FoxJ1 negative cells with multiple cilia among the total cells was less than 0.5%, and they did not show a statistically significant difference between the conditions. Among the total cells, 2.19 ± 0.68%, 2.27 ± 1.11%, and 1.69 ± 0.58% of cells were nestin-positive in control, BMP2, and BMP4 conditions, respectively, with no statistically significant difference (Fig. 7C).

Discussion

We reported here that both BMP2 and BMP4 inhibited ciliogenesis during the ependymal cell differentiation *in vitro*. In our experiments, the number of multi-ciliated cells was lower than that of the previous study (70-80%) (Delgehyr et al. 2015) in 2 weeks of culture without fetal bovine serum. Because the progress of cell differentiation reached a plateau in 14 days in medium without serum (Fig. 2A, B), the lower number of multi-ciliated cells in our study could not be due to delayed differentiation. The deviation in the number of differentiated cells between the previous study and our study may be due to material or technical variations, such as differences in reagent supplier or technical procedures in cell culture.

Our result showed that both BMP2 and BMP4 strongly suppressed the formation of multi-ciliated cells during ependymal cell differentiation (Fig. 3A, B), consistent with the previous study which showed that ectopic expression of Smad6 and Smad7, intracellular inhibitory factors of the BMP signaling pathway, induced ependymal-like ciliated cells from mouse embryonic stem cells (Nishimura et al.

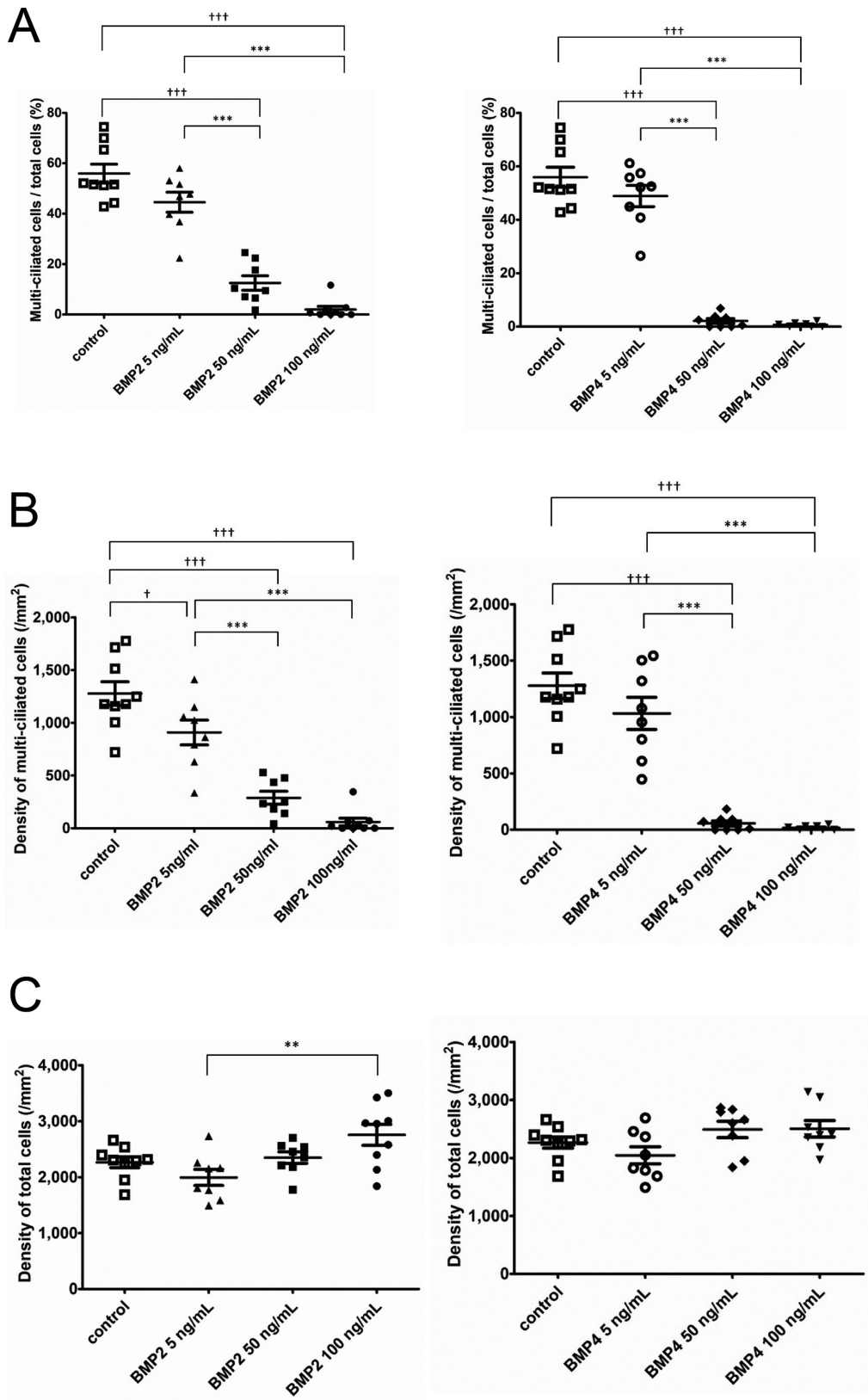

Fig. 3. Effect of bone morphogenetic proteins (BMPs) on cell differentiation.
The number of multi-ciliated cells per total cells (A), the density of multi-ciliated cells (B), and the density of total cells (C) in different BMPs concentrations and medium with vehicle (control) are shown. The same control data is used for analyses of both BMP2 conditions and BMP4 conditions. n = 8 or 9. Data are mean ± SEM. "†" indicates a significant difference between day 0 and the other days. "*" indicates significant difference between days except day 0.
$^{*}p < 0.05$, $^{**}p < 0.01$, ***, $^{\dagger\dagger\dagger}p < 0.0001$.

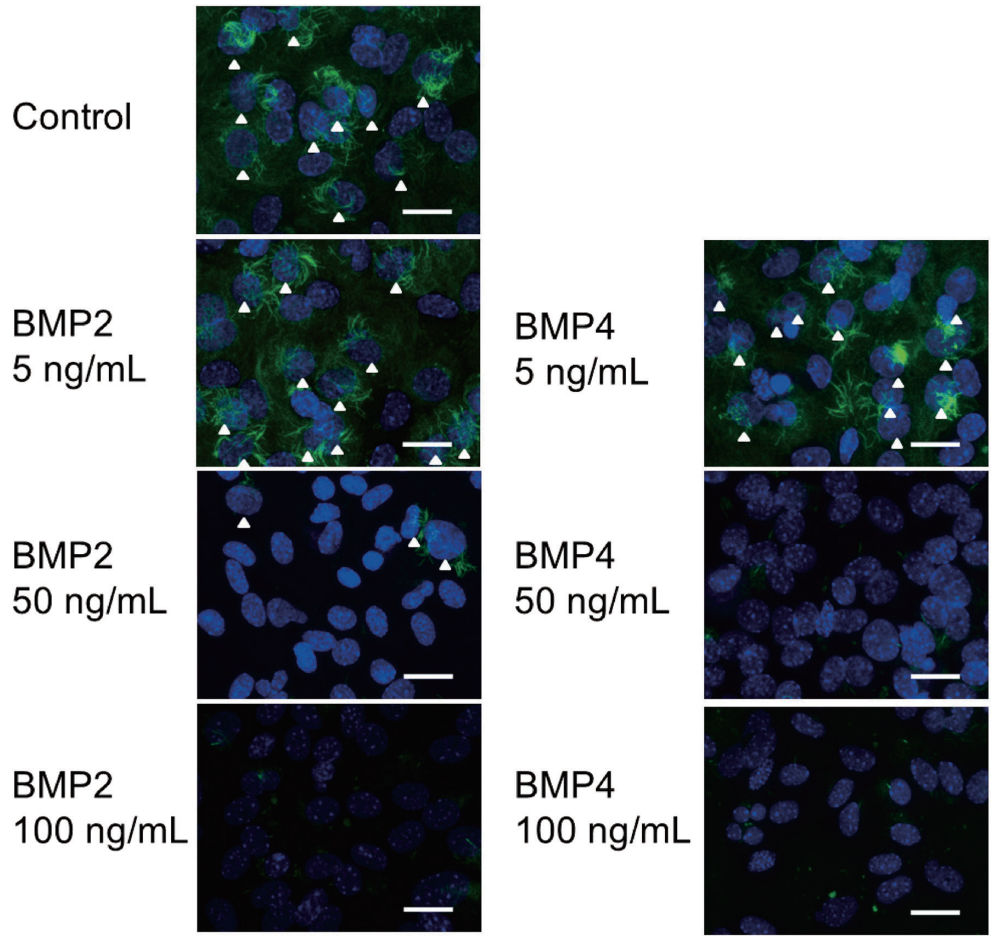

Fig. 4. The cells cultured in different BMPs concentrations and medium with vehicle (Control). A cilia marker, β-Tubulin IV, is stained in green. The nuclei are counterstained with DAPI in blue. Arrowheads indicate multi-ciliated cells. Scale bar: 20 μm.

2010). The cells cultured with BMPs did not express multiple cilia, while FoxJ1, a nuclear marker for ciliated cells, was expressed even in the cells without cilia. The results may suggest that BMPs inhibit ciliogenesis, but do not affect the cell fate of ependymal cells.

In the initial cell population before induction of differentiation, the cells had neither multiple cilia nor nestin expression, while 53.66% of cells were FoxJ1 positive. These data suggested that the initial population did not possess the property of stem cells, and about half of the cells were already fated to differentiate to multi-ciliated cells. Although the cells were nestin negative at this stage, some cells were nestin-positive in the later stages, as shown in other experiments in this study. In the BrdU labeling experiment, a small population of cells that underwent mitosis was observed, and a part of them was nestin positive. These data suggested that the initial cell population may contain a limited number of stem cells with weak expression of nestin, and nestin expression increased during the culture in the medium without fetal bovine serum. The number of cells that underwent mitosis did not show a statistically significant difference in the BMP conditions compared to the control condition, suggesting that BMPs do not affect cell proliferation.

Since the exact local concentration of BMPs in the site of ependymal differentiation *in vivo* has not been known, we followed here the concentrations used in the previous cell culture studies (Gross et al. 1996; Lim et al. 2000). The Allen Developing Mouse Brain Atlas (https://developingmouse.brain-map.org/) showed BMP1, BMP4, and BMP5 appeared to be expressed in the choroid plexus but not in the ventricular walls at embryonal day 18.5. Only BMP4 appeared to be expressed in the ventricular walls at postnatal day 4, and subsequently, BMP1, BMP2, BMP4, BMP6, BMP7, BMP8a, BMP10, and BMP15 appeared to be expressed in the ventricular wall on postnatal day 14. In addition, there are evident reports that BMP2 and/or BMP4 is concentrated at the ventricular zone surface of the mouse brain in the embryonic stage (Li et al. 1998) and the adult (Lim et al. 2000). These may suggest that progenitor cells are continuously exposed to BMPs during ependymal differentiation.

Although the physiological significance of ciliogenesis regulation in ependymal cells by BMPs remains unknown, our study suggests that inhibition of the BMP signaling pathway might be essential for ciliogenesis during the epen-

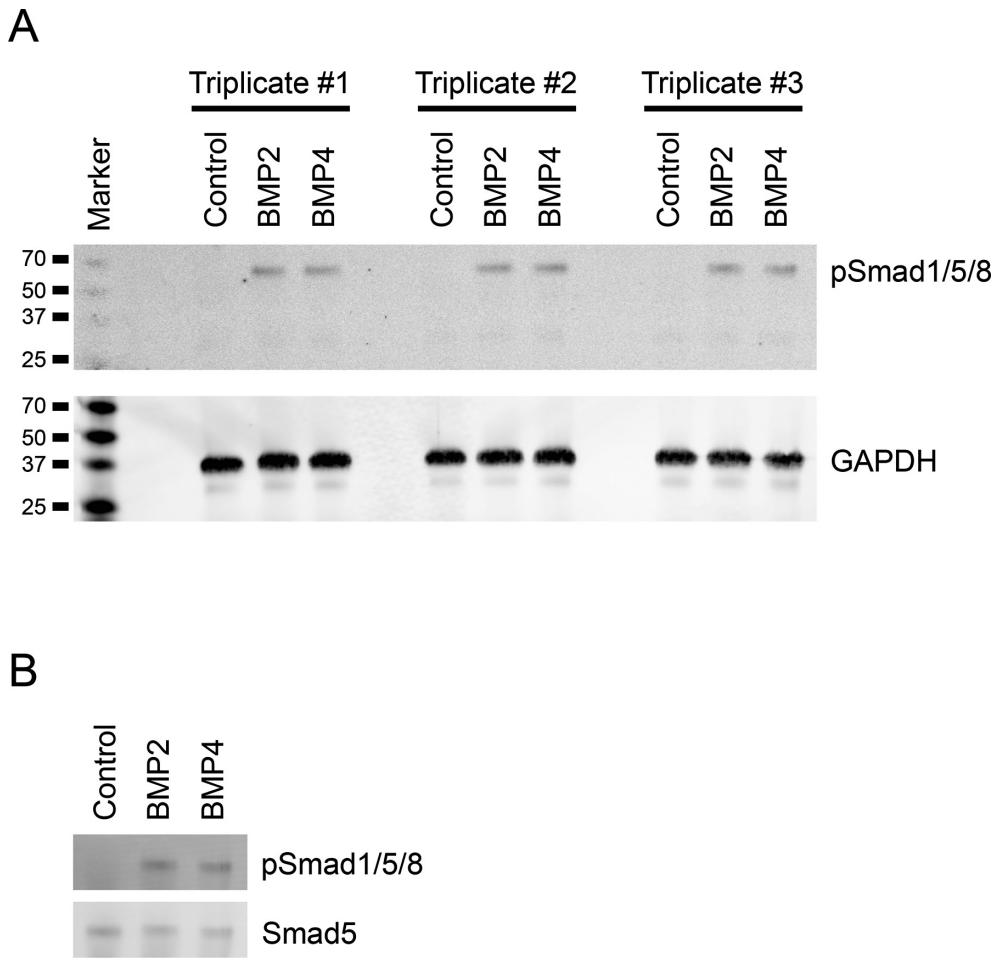

Fig. 5. Phosphorylation of Smad1/5/8 proteins by BMP treatment in the primary ependymal cells.
A. Signals of phosphorylated Smad1/5/8 (pSmad1/5/8) were detected in BMP2 or BMP4-treated cells (BMP2 or BMP4) but not in the control cells (Control). GAPDH was used as an internal control.
B. The signal of non-phosphorylated Smad5 was similar in each condition.

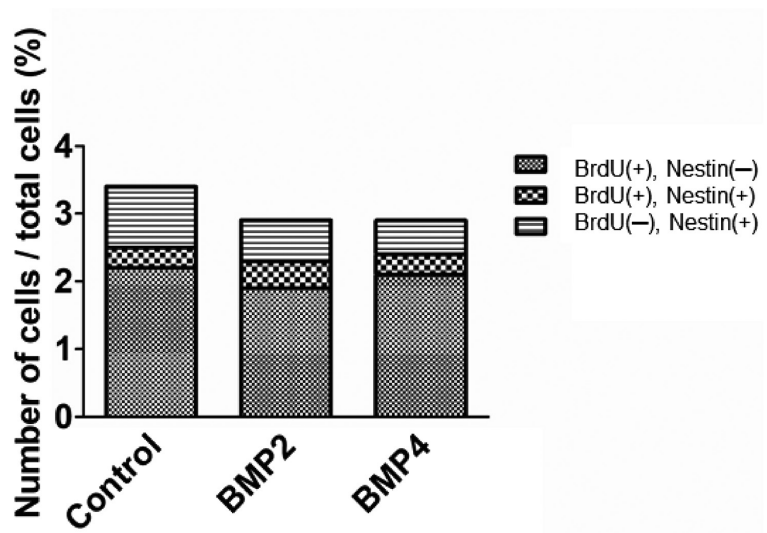

Fig. 6. Cell proliferation during ependymal differentiation.
Mean number of BrdU-positive Nestin-negative cells (BrdU(+), Nestin(–)), BrdU-positive Nestin-positive cells (BrdU(+), Nestin(+)), BrdU-negative Nestin-positive cells (BrdU(–), Nestin(+)) among total cells in each condition are shown. 50 ng of BMP2 or BMP4 is supplemented in 1 mL medium in the BMP conditions (n = 3).

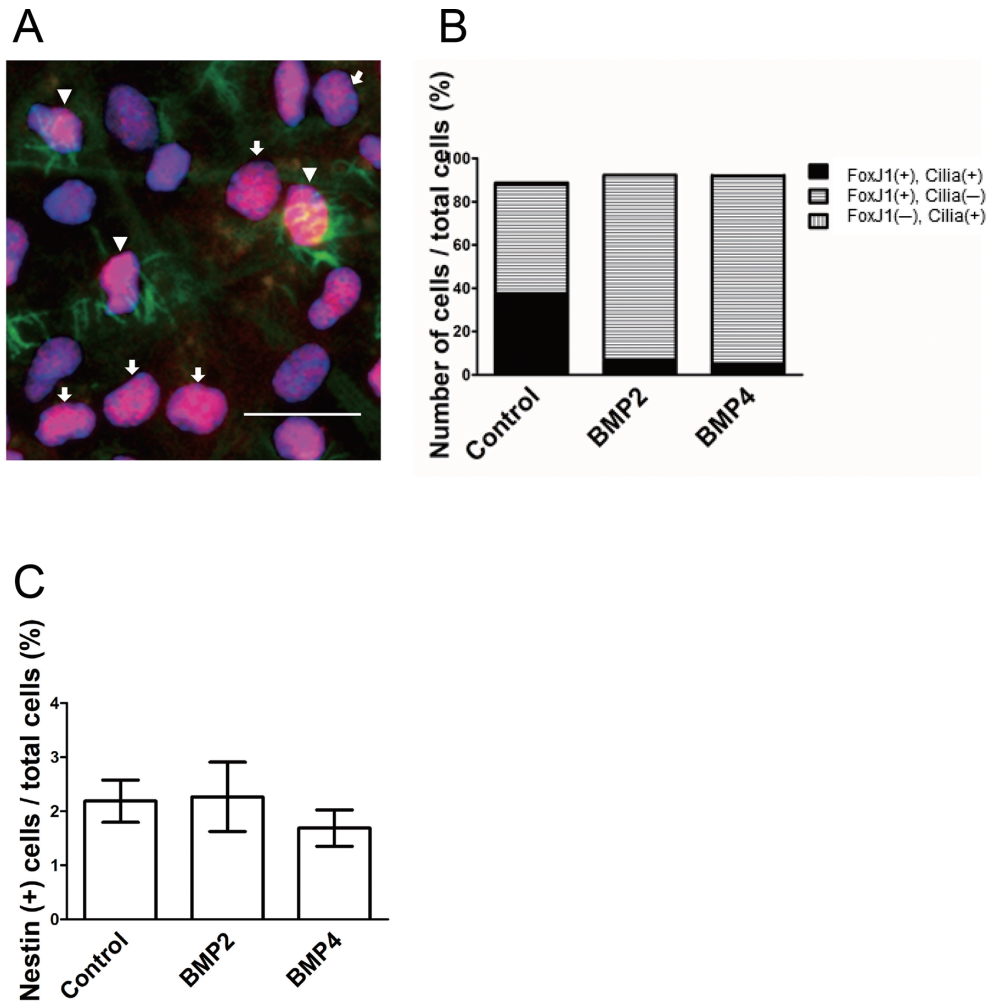

Fig. 7. FoxJ1 and nestin expressions in the cultured cells.

A. Representative image of cells cultured in medium without BMPs (control condition) for 14 days. A cilia marker, β-Tubulin IV, is stained in green, and FoxJ1, a nuclear marker for ciliated cells is stained in magenta. The nuclei are counterstained with DAPI in blue. Arrows indicate FoxJ1-positive cells without multiple cilia, and arrowheads indicate FoxJ1-positive cells with multiple cilia. Scale bar: 20 μm.

B. The number of FoxJ1-positive cilia-positive cells (FoxJ1(+), Cilia(+)), FoxJ1-positive cilia-negative cells (FoxJ1(+), Cilia(–)), FoxJ1-negative cilia-positive cells (FoxJ1(–), Cilia(+)) among the total cells in each condition are shown. The number of FoxJ1-negative cilia-positive cells among the total cells was less than 0.5%. 50 ng of BMP2 or BMP4 is supplemented in 1 mL medium in the BMP conditions. n = 3.

C. The number of nestin-positive cells among the total cells in each condition are shown. 50 ng of BMP2 or BMP4 is supplemented in 1 mL medium in the BMP conditions. Data are mean ± SEM; n = 3.

dymal cell differentiation in consideration of the situation that progenitor cells are exposed to BMPs during ependymal differentiation. Extracellular BMP antagonists such as Noggin (Lim et al. 2000; Liu and Niswander 2005) or intracellular inhibitory factors of the BMP signaling pathway, Smad 6 or Smad7 (Liu and Niswander 2005), could be expressed by ependymal progenitor cells during ependymal differentiation. Indeed, the Allen Developing Mouse Brain Atlas shows that Noggin and Smad7 are clearly expressed in the ventricular wall at postnatal day 14.

In conclusion, the present *in vitro* results suggest that inhibition of the BMP signaling pathway might be essential for ciliogenesis during the ependymal cell differentiation *in vivo*. Activation of the BMP signaling pathway inhibits ciliogenesis but does not affect cell fate, suggesting that inhibition of the BMP signaling pathway might be critical for the onset of ciliogenesis in the ependymal cell differentiation during brain development.

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Conflict of Interest

The authors declare no conflict of interest.

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