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Robust Enhancement of Neural Differentiation from Human ES and iPS Cells Regardless of their Innate Difference in Differentiation Propensity

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Abstract Our analyses of three human induced pluripotent stem cell (hiPSC) and six human embryonic stem cell (hESC) lines showed marked variability in differentiation potential into specific lineages, which often hampers their differentiation into specific cell types or cell lineages of interest. Simultaneous inhibition of both Activin/Nodal and BMP pathways with small molecules, SB431542 and dorsomorphin (DM), respectively, promoted significant neural differentiation from all human pluripotent stem cell (hPSC) lines tested, regardless of their differentiation

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propensity. On the contrary, differentiation into other cell lineages and the number of undifferentiated cells were significantly reduced after differentiation by the dual inhibition. These results demonstrate that innate differentiation propensity of hPSCs could be overcome, at least in part, by modulation of intracellular signaling pathways, resulting in efficient generation of desirable cell types, such as neural cells.

Keywords Pluripotent stem cell · Differentiation propensity · Neural induction · Cell signaling · Small molecule

Introduction

Due to their pluripotent nature, human pluripotent stem cells (hPSCs) including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) could serve as an inexhaustible source of diverse cell types for future cell replacement therapies. Furthermore, hPSCs are useful for screening drugs and exploring early embryonic development as cellular model systems. In all cases, efficient and strict differentiation of hPSCs into specific cell types of interest is a prerequisite.

A recent report claimed a very important issue in stem cell research that each hESC line has its own differentiation inclination toward a specific cell lineage [1]. In the light of the importance of strict differentiation of hESCs for their successful uses, this issue of differentiation propensity among different hESC lines awaits further corroboration by subsequent studies from other groups. In this study, we showed that hESCs generated from various institutions display their own distinctive propensity to be differentiated into certain cell lineages. Furthermore, we demonstrated for the first time that each hiPSC line also retains a unique differentiation propensity.

The innate differentiation propensity of hPSCs often negatively affects differentiation into desirable cell lineages. Therefore, a thorough examination of the differentiation propensity of all hESCs and hiPSCs in advance is highly advisable so that appropriate cell lines can be chosen for specific therapeutic applications. Since the screening for the innate differentiation propensity of all hPSCs is laborious, time-consuming, and costly, it would be much beneficial if there is a way to induce differentiation of any hPSC line into desirable cell types, regardless of its innate differentiation propensity.

Derivation of specific cell types from hPSCs becomes more significant due to the recent approval of the world's first clinical trial using hESCs as well as rapid development of hiPSC research field, which make both hESCs and hiPSCs a realistic option for cell replacement therapy. In this study, we demonstrate that hESC and hiPSC lines, regardless of their own differentiation propensity, can be efficiently coaxed into neural lineage by modulating intracellular signaling pathways such as Activin/Nodal and BMP pathways with small molecules.

Materials and Methods

hESC and hiPSC Culture

The 6 hESC lines used in this study, H9 (P31-45, WiCell Inc, Madison, USA), Miz-hES4 (P67–75) and Miz-hES6 (P34–45) (MizMedi Hospital, Seoul, Korea), CHA-hES3 (P88–93, CHA Hospital, Seoul, Korea), SNU-hES3 (P30–36) and SNU-hES16 (P71–76) (Seoul National University Hospital, Seoul. Korea), were routinely cultured in DMEM/F12 medium supplemented with 20% KSR (Invitrogen, Carlsbad, USA), 1x non-essential amino acid (Invitrogen), 0.1 mM beta-mercaptoethanol (Sigma, St. Louis, USA), and 4 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen). Most hESC cell lines were grown on the layer of mitotically-arrested mouse embryonic fibroblasts (MEFs), except SNU-hES3 and 16 which were cultured on STO (ATCC, Manassas, USA) feeder cells. hESC colonies were transferred onto a fresh feeder layer in every 5–7 days by mechanical passaging as previously described [2]. Three human iPSCs, dH1f-iPS2-2, MSC-iPS2-3, and BJ1-iPS12 [3] were from Dr. George Daley's lab at Harvard Medical School and cultured in the same condition as hESCs.

Spontaneous Differentiation of hPSC and Subsequent Derivation of Neural Cells and Dopaminergic Neurons

EB formation from hESC and hiPSC colonies was initiated by detaching the colonies from feeder cells by treatment of 2 mg/ml of type IV collagenase (Invitrogen) for 30 min and transferring the colonies to Petri dish containing normal hESC culture medium without bFGF (EB medium). For spontaneous differentiation, the EBs were cultured for 10 days with medium change every 2 days. To examine the effect of dorsomorphin (DM) (also known as Compound C, Sigma) and SB431542 (Calbiochem, San Diego, USA) in spontaneous differentiation, various concentrations of the two small molecules were added in the EB medium during the 10-day EB culture. The expression of several markers was analyzed by qRT-PCR and immunocytochemistry.

Neural precursor cells (NPCs) formed in EBs were expanded in suspension culture in N2 medium (DMEM-F12 & 1×N2 supplement, Invitrogen) containing bFGF (20 ng/ml, Invitrogen) for additional 8–10 days with changing of medium every other day. The expanded NPCs

were then triturated and grown on Matrigel (BD Scientific, Bedford, USA)-coated cover-slips either in N2 medium supplemented with 2% FBS (Invitrogen) for additional 4 weeks (to examine the tri-potency of NPCs) or in N2 medium containing 500 ng/ml of Sonic Hedgehog (SHH) (R&D Systems, Minneapolis, USA) and 100 ng/ml of FGF8 (R&D Systems) (to induce dopaminergic commitment as previously reported [4]). Dopaminergic maturation was performed by the treatment of 20 ng/ml brain-derived neurotrophic factor (R&D Systems), 20 ng/ml glial cell line-derived neurotrophic factor (R&D Systems), and 200 μM ascorbic acid (Sigma) in DMEM/F12 media supplemented with N2.

Directed Neural Differentiation of hPSCs

Directed differentiation of hPSCs into neural lineage cells was performed using the previously reported method with minor modification [5]. Briefly, EBs were cultured in suspension for 4 days in EB medium with and without 5 μM DM and 5–10 μM SB431542, and then cultured attached on Matrigel-coated dish in N2 media supplemented with 20 ng/ml bFGF for additional 6 days. Samples were analyzed by colony counting, immunocytochemistry and qRT-PCR.

Immunostaining and Quantitative Analysis

Cells were fixed in 4% para-formaldehyde/PBS solution for 30 min. EBs were also fixed in the same fixative for 1 h, cryoprotected with 30% sucrose, frozen in O.C.T. compound (Tissue Tek, Torrance, USA), and sectioned at 10 μm thickness with a cryostat. The sections were permeabilized with 0.1% Triton X-100/PBS (for intracellular markers), blocked with 5% normal donkey serum for 1 h at room temperature, and then treated with primary antibodies at 4°C over-night. Primary antibodies used in our study were as follows: Oct4 (1:200, Santa Cruz Biotechnology, Santa-Cruz, USA); SSEA4 (1:500, Santa Cruz Biotechnology); Sox1 (1:200, Millipore, Billerica, USA); Pax6 (1:200, DSHB, Iowa, USA), nestin (1:1,000, Millipore); α-fetoprotein (AFP) (1:100, Santa Cruz Biotechnology); Tuj1 (1:1,000, Covance, Berkeley, USA); GFAP (1:300, Millipore), and O4 (1:200, R&D systems). After the primary antibody incubation, appropriate fluorescence (Alexa-Fluor®-488 or 594)-tagged secondary antibodies (Molecular Probes, Eugene, USA) were used for visualization. Cells were treated with DAPI (4', 6-diamidino-2-phenylindole, Vector, Burlingame, USA) for 5 min during the staining procedure to visualize the nuclei. Cells generated after DA differentiation were also fixed in 4% para-formaldehyde/PBS solution and subjected to subsequent incubations with appropriate primary and secondary antibodies as described above. Cell images were captured with Olympus IX71 microscope and DP71 digital camera, and analyzed by Image-Pro Plus ver5.1 (Media Cybernetics, Silver Spring, USA). Quantitative evaluation was performed by counting immuno-labeled cells or colonies from three independent experiments. Values were expressed as means ± s.e.m. Student t-test or one-way ANOVA test using the SPSS software Version 12.0 was used to determine statistical significance.

Quantitative RT-PCR (qRT-PCR) Analyses

Total RNAs were extracted using a Easy-Spin® total RNA purification kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions and then 1 μg of the total RNAs were reverse transcribed with Power cDNA synthesis kit (iNtRON Biotechnology). qRT-PCR was performed using SYBR Premix Ex Taq ™ (Takara Bio Inc, Shiga, Japan) and the reaction was carried out using the My-iQ or CFX96 Real-Time System (Bio-Rad, Hercules, USA) under the following conditions; (step 1) 1 min at 95°C; (step 2) 40 cycles of 20 s at 95°C, 20 s at 63°C, and 20 s at 72°C; (step 3) final extension for 1 min at 72°C. Expression values (Ct values) of specific marker genes were collected and normalized according to those of β-actin, and then the normalized expression levels of the markers were compared between chemical-treated samples and vehicle-treated control samples according to the ΔΔCt method [6]. All of the data was confirmed by at least three independent experiments. The primer sequences are listed in Supplementary Table 1.

Western Blot Analyses

To confirm if treatment of hESCs with DM inhibits BMP signaling pathway, H9 cells were treated with various concentrations of DM or 1 μg/ml of noggin (R&D Systems) for 30 min, followed by another 30-minute treatment with BMP4 (50 ng/ml, R&D Systems). The cells were then immediately lysed in RIPA buffer (Sigma) containing both phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and subjected to Western blot analysis.

To compare the basal BMP signaling activities between H9 and Miz-hES4 cells, phosphorylation level of Smad1/5/8 in the cells were examined by measuring the ratio of phospho (p)- and total Smad1/5/8 by Western blot. In addition, EBs derived from H9 and Miz-hES4 cells in the presence and absence of DM (5 μM) and SB431542 (10 μM) were also examined for the level of Smad1/5/8 phosphorylation at day 4 of EB differentiation.

For Western blot analyses, total protein (30 μg) was electroporesed on 10% polyacrylamide gel containing

sodium dodecyl sulfate and immediately transferred onto the nitrocellulose membrane (Bio-Rad, Hercules, USA). Blots were incubated overnight at 4°C with the appropriate primary antibodies, followed by incubation with secondary antibody for 1 hr at room temperature, and then visualized using ECL substrate solution (Pierce, Rockford, USA). The following primary antibodies were used for our Western blot analyses: p-Smad1/5/8, p-Smad2/3 (Cell Signaling Technology, Danvers, USA), Smad1/5/8, Smad2/3 (Santa-Cruz Biotechnology), and β-actin (Sigma).

Results

Differences in Differentiation Propensity among hiPSC as well as hESC Lines

One of the critical issues to be resolved for clinical applications of hESCs is a strict differentiation of the cells into desirable cell types. A recent study claiming that each hESC line has different innate differentiation propensity [1] emphasizes the need for careful choice of hESC lines depending on the nature of applications. Since this issue of differentiation propensity is not a trivial matter, further close scrutiny will be needed, hopely with some solution to overcome the problem. To resolve this issue, we set out to examine total nine hPSC lines (six hESC lines established by four institutions and three hiPSC lines) for their differentiation propensity under spontaneous differentiation condition. Each hPSC line was differentiated into EBs for 10 days in EB medium without any differentiation inducing molecule, and then expression level of markers specific for three germ layers (Sox1, neuroectoderm; Brachyury, medoderm; GATA4, endoderm) and undifferentiated cells (Oct4) were assessed by qRT-PCR.

Our results demonstrated significant difference in differentiation propensity among the six hESC lines tested, which is in consistent with the previous report [1] (Fig. 1). H9 and Miz-hES6 cells retain higher potential to differentiate into neuroectodermal lineage than the other hESC lines. On the other hand, Miz-hES4 cells are prone to become meso/endodermal cells, while SNU-hES3, SNU-hES16, and

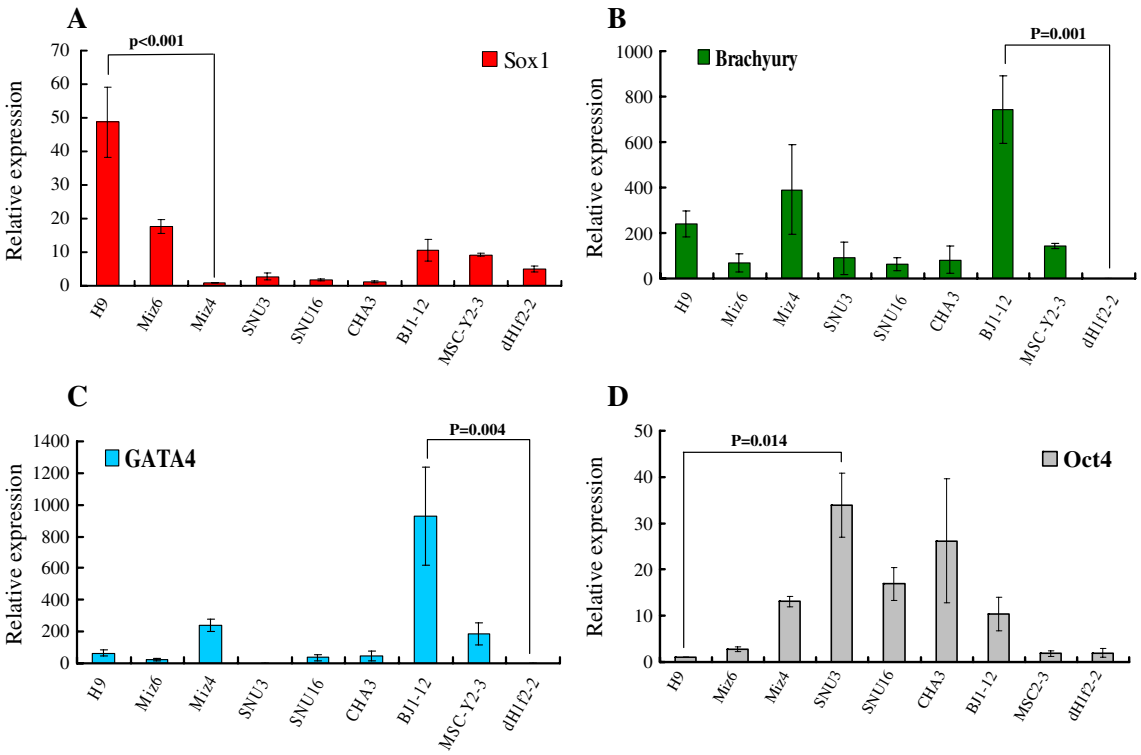

Fig. 1 Variations in differentiation propensity among hPSCs. Total 6 hESC lines (H9, Miz-hES4 and 6, SNU-hES3 and 16, CHA-hES3) and 3 human iPSC lines (BJ1-iPS12, MSC-iPS2-3, dH1f-iPS2-2) were examined for their differentiation propensity. After 10 days of spontaneous differentiation in EB medium, expression levels of representative neuroectoderm (Sox1) **a**, mesoderm (Brachyury) **b**, endoderm (GATA4) **c**, and undifferentiation markers (Oct4) **d** were assessed by qRT-PCR. The y-axis represents means ± s.e.m of relative expression level of each gene over the lowest one (arbitrarily designated as 1) among tested cell lines. Statistical significance was estimated using one-way ANOVA (analysis of variance) test with multiple comparisons among cell lines. To reduce a type I error rate, we applied Bonferroni correction as Post Hoc. Miz6, Miz-hES6; Miz4, Miz-hES4; SNU3, SNU-hES3; SNU16, SNU-hES16; CHA3, CHA-hES3; BJ1-12, BJ1-iPS12; MSC2-3, MSC-iPS2-3; dH1f2-2, dH1f-iPS2-2

CHA-hES3 cell lines are less efficiently committed to differentiation than the other hESC lines (Fig. 1).

In addition, we noticed that hiPSC lines also displayed different potential to differentiate into specific cell lineages (Fig. 1). The three hiPSC lines tested in our analysis, BJ1-iPS12, MSC-iPS2-3, and dH1f-iPS2-2, have been derived from BJ1 neonatal fibroblasts, mesenchymal stem cells, and H1 hESC-derived fibroblasts, respectively [3]. Among the hiPSC lines, BJ1-iPS12 has differentiation propensity toward meso/endodermal lineages as judged by significant expression of Brachyury (medodermal marker) and GATA4 (endodermal marker) (Fig. 1).

Taken together, our results indicate that not only hESCs but also hiPSCs retain their own unique differentiation propensity.

Effect of Blocking BMP Pathway using a Small Molecule, Dorsomorphine (DM), on Differentiation of hESCs

We have been interested in efficient derivation of neural cells from hPSCs for future cell therapy of neurological diseases. In this regard, innate differentiation propensity unfavorable to neuroectodermal lineage might be an obstacle in obtaining pure population of neural cells from some hPSC lines. Therefore, we attempted to establish a method that efficiently drives all hPSC lines toward neural lineage (i.e. formation of neural precursor cells (NPCs)), regardless of their innate differentiation propensity.

Our strategy focused on manipulating cell signaling pathways critically involved in neural induction during early embryonic development. Since inhibition of bone morphogenetic protein (BMP) signaling pathway was shown to enhance neural induction during early embryonic stage [7, 8], we examined if blocking the signaling using a small molecule would promote neural differentiation of hPSCs while repressing differentiation into the other lineages. To this end, H9 cells were treated with dorsomorphin (DM) during their spontaneous differentiation. DM was recently identified as a selective inhibitor of the BMP type I receptors, activin receptor-like kinases (ALKs) 2, 3, and 6, and was shown to block BMP-mediated phosphorylation of Smad1/5/8 in zebrafish embryos [9].

We first assessed the effectiveness of DM as well as its optimal dose for the treatment. Undifferentiated H9 cells were first treated with DM (0.1 μM–5 μM) or DMSO (vehicle, control) for 30 min and then with 50 ng/ml of BMP4 for 30 min. Western blot analysis demonstrated that pretreatment with DM effectively blocked BMP4-induced phosphorylation of Smad1/5/8 in a dose-dependent manner, when compared to the control sample (Fig. 2a). We then confirmed that DM (0.1 μM–5 μM) treatment diminished the expression level of Id1 and Id3 genes, the indicators of BMP signaling activity, in dose dependent manner in differentiating EBs (Fig. 2b). Intriguingly, DM-treatment (0.1 μM–5 μM) for 4 days in EB culture increased expression of neural markers such as Sox1, Pax6, and nestin dose-dependently (Fig. 2c), which indicates that inhibition of BMP signaling pathway promotes differentiation of H9 cells toward neural lineage. Collectively, our results demonstrated that DM more efficiently inhibits BMP signaling pathway than a peptide antagonist noggin and the most prominent effect can be seen at 5 μM DM (Fig. 2a-c).

Next, we closely investigated whether the inhibition of BMP pathway by DM sufficiently induced neural differentiation of hESCs while reducing differentiation into the other lineages. In this experiment, H9 cells were differentiated for 10 days in EB medium supplemented with DM (1 and 5 μM), and then expression of representative markers for each germ layer as well as undifferentiated hESCs was examined by qRT-PCR and immunocytochemistry (Fig. 3). DM-treatment during the spontaneous differentiation significantly enhanced the expression of neural markers (Sox1 and nestin) in a dose-dependent manner, while markers for mesoderm (Brachyury and Cerberus), endoderm (GATA4 and alpha-fetoprotein (AFP)) and undifferentiated hESCs (Oct4 and Nanog) tended to decrease (Fig. 3a). In support of this, nestin-positive cells were robustly increased after DM-treatment (Fig. 3b, *top panels*). However, although reduced, the expression of endo/mesoderm- and undifferentiated cell markers were still considerable (Fig. 3a, b), implying that blocking BMP pathway alone is not sufficient to generate highly pure population of neural cells with minimal contamination of endo/mesodermal and undifferentiated cells. This conclusion prompted us to look for additional signaling pathway the inhibition of which would further enhance the differentiation of hESCs toward neural lineage.

Simultaneous Inhibition of BMP and Activin/Nodal Pathways using DM and SB431542, Respectively, Induces Highly Pure Population of Neural Cells from hESCs

Activin/Nodal pathway has been known to play a pivotal role during early embryonic development by inducing endodermal and mesodermal differentiation [10], while suppressing differentiation into neuroectodermal lineage [11, 12]. In addition, a recent report demonstrated that Activin/Nodal signaling is also important for maintaining stemness of hESCs [13, 14]. Therefore, we postulated that interfering of Activin/Nodal signaling would drive differentiation of hESCs favorably toward neuroectoderm rather than other lineages and undifferentiated cells.

To test this hypothesis, we blocked Activin/Nodal signaling pathway, in addition to BMP pathway, by treating with a small molecule antagonist called SB431542 (5 or 10 μM) during spontaneous differentiation of H9 cells. SB-431542 was characterized as a competitive inhibitor of ALKs 4, 5, and 7 implicated in Activin/Nodal signaling. In

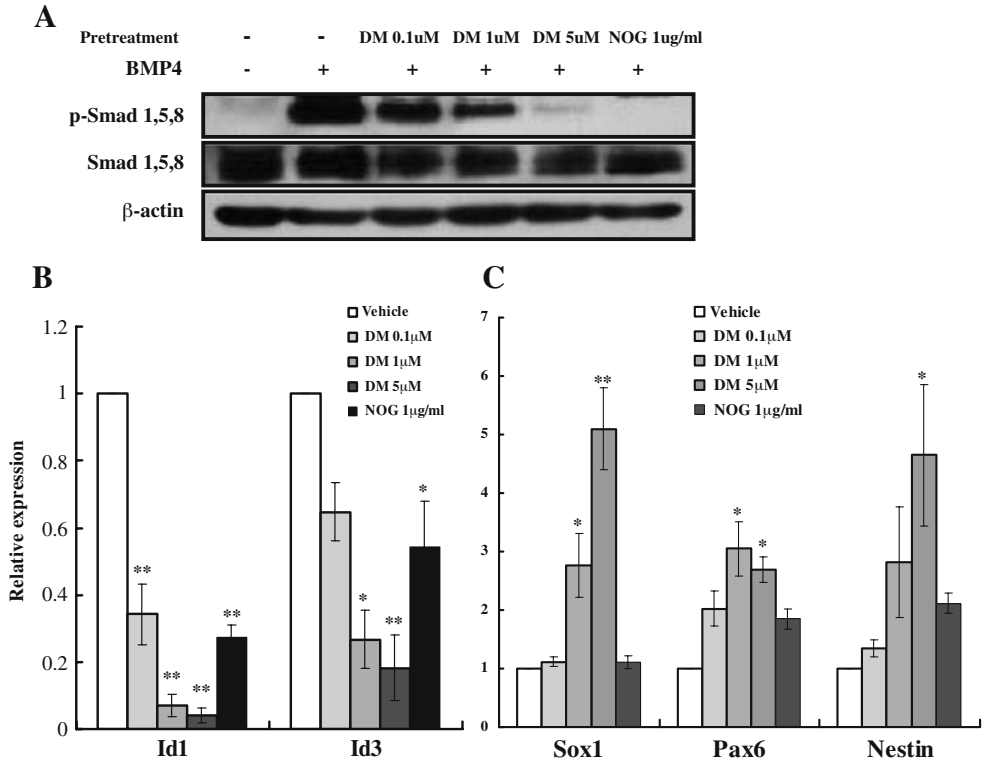

Fig. 2 Treatment of DM effectively inhibited the BMP signaling cascade, resulting in augmented neural differentiation. **a** DM (0.1–5 μM) inhibits BMP-mediated activation of Smad 1/5/8 in hESCs dose-dependently. Thirty μg of total proteins were loaded onto each lane of the gel. **b**, **c** 4 days after spontaneous differentiation in the presence or absence of DM, the expression level of several markers was measured from EBs by qRT-PCR. **b** Expression levels of Id1 and Id3 genes, indicators of BMP signaling activity, was decreased by the treatment of DM (0.1 μM–5 μM) in a dose-dependent manner. One μg/ml of noggin was used as a positive control. **c** Expression of neuroectodermal markers (Sox1, Pax6, and nestin) was increased by the 4-day treatment of DM. The y-axis in the graphs represents the relative expression level of each gene after DM- or noggin-treatment compared to vehicle-treatment. DM, dorsomorphin; NOG, noggin. (* $p<0.05$, ** $p<0.01$ compared with control group, ANOVA test)

detail, SB431542 inhibits ALK4/5/7-mediated phosphorylation of Smad 2/3 and this inhibition prevents Smad2/3 from entering into the nucleus where they function as transcriptional regulators. SB431542, however, has no effect on the ALK family members involved in other signaling pathways. For example, SB431542 does not inhibit ALKs 2, 3, and 6 which phosphorylate Smad1/5/8, critical mediators of BMP signaling pathway [15].

After 10 days of differentiation, the expression of neural markers (Sox1, Pax6, and nestin) was enhanced, while the level of both endoderm (GATA4 and AFP) and mesoderm (Brachyury and Cerberus) markers were dramatically reduced (Fig. 4a). More importantly, markers for undifferentiated hESCs (Oct4 and Nanog) were also greatly reduced (Fig. 4a). The enhanced expression of neural markers (Pax6 and nestin) and reduction of endo/mesodermal markers (AFP and Brachyury) were confirmed by immunocytochemistry (Fig. 4b).

Expression of trophoblast marker genes (GATA2 and GCM1) was decreased by inhibition of either BMP pathway alone (DM-treatment) or both BMP and Activin/Nodal pathways (DM/SB431542-treatment) (Fig. 4c). This result is in line with the previous observation that blocking of Activin/Nodal pathway leads to differentiation of hESCs into trophoblasts only when BMP signal is active [16].

The NPCs generated in our experiments were readily differentiated into dopaminergic (DA) neurons by treatment of SHH and FGF8 [4] (Supplementary Fig. 1c), indicating that NPCs generated by simultaneous inhibition of BMP and Activin/Nodal pathways could be a useful cell source for cell therapy for neurologic diseases. In addition, all neural cell types, neurons (Tuj1+), astrocytes (GFAP+), and oligodendrocytes (O4+), were produced from the NPCs (Supplementary Fig. 1a, b), suggesting that the NPCs generated by DM/SB431542-treatment were multipotent cells that could give rise to all neural cell types.

In summary, our data suggested that inhibition of both BMP and Activin/Nodal signaling pathways led to efficient and exclusive neural induction from H9 cells. These results are consistent with the recent work showing that simultaneous and continued suppression of BMP and Activin/

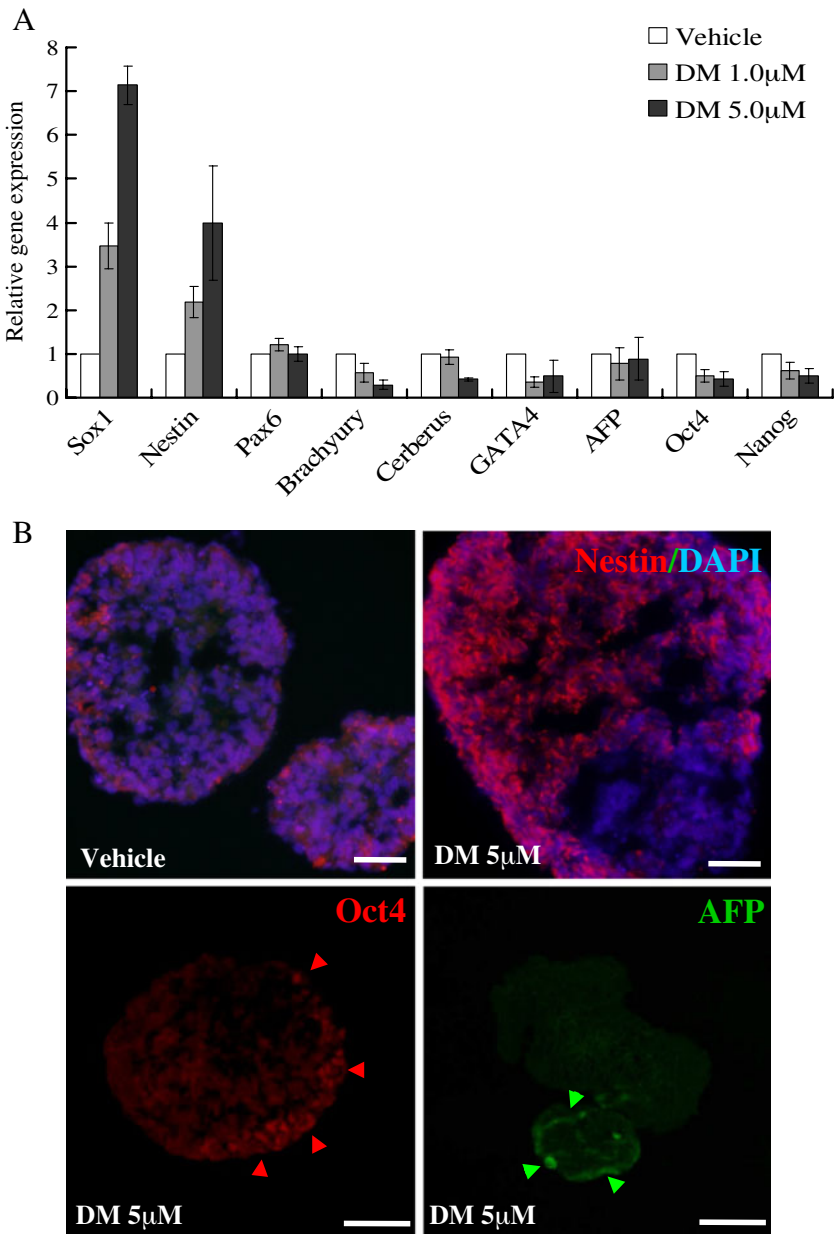

Fig. 3 Differentiation of hESCs (H9) into neuroectodermal lineage by modulation of BMP pathway. **a** Addition of DM in EB medium for 10 days during spontaneous differentiation of H9 cells enhanced the expression of neuroectodermal markers (Sox1, Nestin), while it suppressed that of mesodermal (Brachyury, Cerberus), endodermal (GATA4, AFP) and undifferentiation markers (Oct4, Nanog). **b** Neuroectodermal cells (nestin-positive) were greatly increased after 10-day treatment with DM (*top panels*). Although reduced, undifferentiated (Oct4-positive; red arrowhead) and endodermal cells (AFP-positive; green arrowhead) were still detected in sections of EBs treated with 5 μM DM for 10 days (*lower left and right panels, respectively*). The y-axis in graph **a** represent fold changes in gene expression between chemical-treated and control samples from three independent experiments; DM, dorsomorphin. Scale bar: 50 μm

Nodal signaling is required for neural induction in *Xenopus* embryo development [17].

Simultaneous Inhibition of BMP and Activin/Nodal Signals Induces Efficient Generation of NPCs from Both hESC and hiPSC Lines Regardless of their Innate Differentiation Propensity

One important question to address is whether the simultaneous treatment of DM and SB431542 could efficiently direct the fates of all hESC and hiPSC lines toward neural lineage, regardless of their innate differentiation propensity. To address this question, nine hPSC lines (six hESC and three hiPSC lines) were differentiated through EB formation in the presence of both DM (5 μM) and SB431542 (10 μM) for 10 days. Our qRT-PCR analyses demonstrated that treatment with DM and SB431542 significantly enhanced neural induction with concomitant reduction of cells of the other lineages (Fig. 5a). Interestingly, the fold increase of neural marker expression between control (vehicle (DMSO)-treated cells) and DM/SB431542-treated cells were much higher in the cell lines that had innate differentiation inclination unfavorable to neural lineage; these were Miz-hES4, SNU-hES3, SNU-hES16, CHA-hES3, and BJ1-iPS12 cells (Figs. 1 and 5a). Immunocytochemical analyses also clearly demonstrated that more cells expressed nestin, a neural precursor marker, when both BMP and Activin/Nodal signaling pathways were suppressed by the

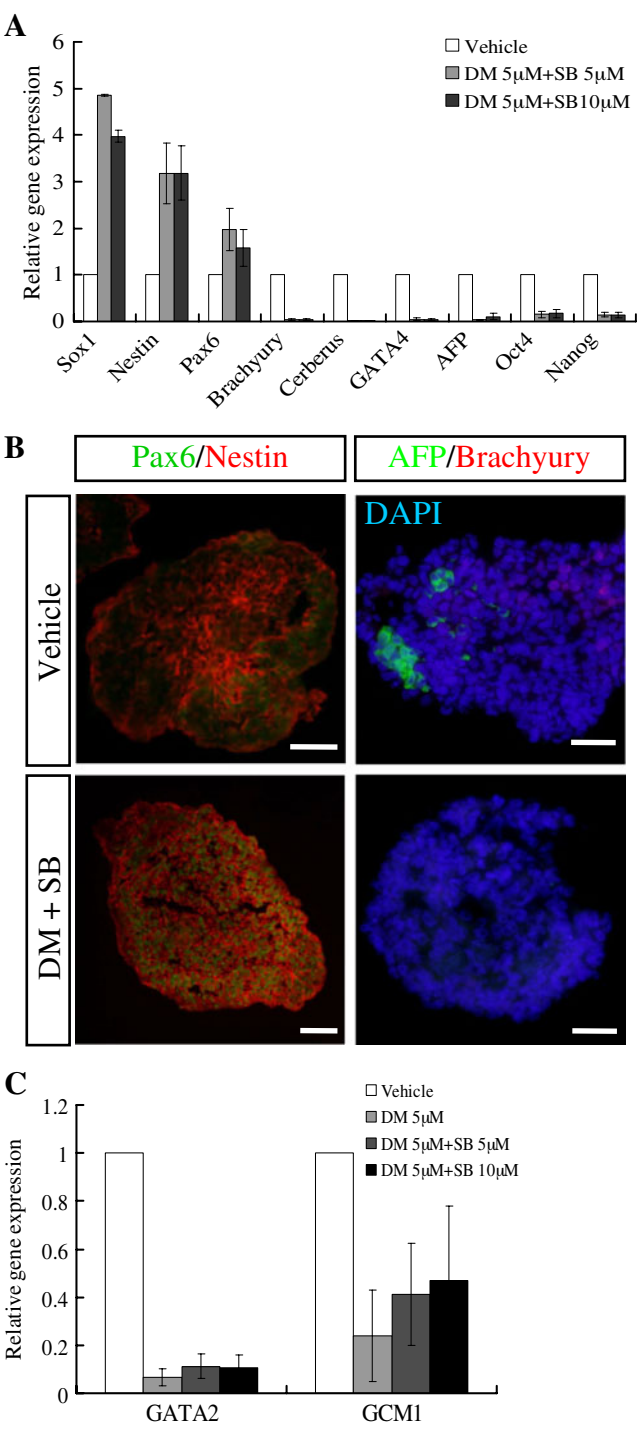

Fig. 4 Differentiation of hESCs (H9) into neuroectodermal lineage by modulation of both BMP and Activin/Nodal signal pathways. **a** Simultaneous treatment with DM and SB431542 for 10 days of spontaneous differentiation enhanced the expression level of neural markers. On the contrary the expression of markers of other lineage and undifferentiation cells was greatly reduced. **b** Immunocytochemical analyses confirmed that expression of Pax6 and nestin was enhanced, while the expression of AFP and Brachyury was reduced after treatment with DM and SB431542, compared with vehicle (DMSO)-treated EBs. **c** EBs were cultured for 10 days with or without DM (5 μM) and SB431542 (5 μM–10 μM) and expression level of two representative trophoblast markers (GATA2 and GCM1) was examined by qRT-PCR. The result indicates that simultaneous inhibition of BMP and Activin/Nodal signaling pathways also suppressed the differentiation of hESCs into trophoblast. The y-axis in graph **a** and **c** represent fold changes in gene expression between chemical-treated and control samples from three independent experiments; DM, dorsomorphin; SB, SB431542. Scale bar: 50 μm

small molecules (Fig. 5b). In consistent with the qRT-PCR results, increase in expression of nestin, a neural marker, by the treatment with DM and SB431542 was much greater when hPSC has lower differentiation propensity toward neuroectodermal lineage (Figs. 1, 5a and b). These observations indicate that blocking both BMP and Activin/Nodal signaling pathways enhances neural formation from hPSCs regardless of their innate differentiation propensity. Additionally, we have not detected any undifferentiated cells after the DM/SB431542-treatment by immunocytochemistry (data not shown).

It is plausible that different differentiation propensity among various hPSC lines may reflect on the basal level of intracellular signaling activity critically involved in cell fate determination. To examine this possibility, we investigated BMP signaling activity between H9 and Miz-hES4 cells, the two cell lines that displayed the most significant difference in neural differentiation propensity (Fig. 1a). During both undifferentiated and spontaneously differentiated state, intensity of phosphorylated form of Smad1/5/8 was higher in Miz-hES4 than H9 cells (Fig. 5c, *top panel, the first four lanes*), indicating that Miz-hES4 cells retain higher BMP signaling activity than H9 cells in those conditions. Interestingly, the treatment of DM and SB431542 dramatically reduced the level of p-Smads in both H9 and Miz-hES4 cells to a minimal level (Fig. 5c, *top panel, the last two lanes*), which explains highly efficient neural differentiation from both cell lines by the treatment.

Different Differentiation Propensity among hPSC Lines after Directed Neural Differentiation and Enhanced Generation of NPCs by Simultaneous Inhibition of BMP and Activin/Nodal Signals

Our results so far indicate that different differentiation propensity is evident among hPSCs after spontaneous differentiation, and efficient neural cell formation could be achieved by simultaneous inhibition of BMP and Activin/Nodal signaling pathways regardless of their innate differentiation propensity. Next, we examined if similar results were obtained when hPSCs were forced to differentiate into the neural lineage using a directed differentiation protocol. For this experiment, we chose H9 and Miz-hES6 lines with strong propensity towards formation of neuroectodermal lineage, and Miz-hES4 and BJ1-iPS12 lines with weak neuroectodermal differentiation propensity (Fig. 1a).

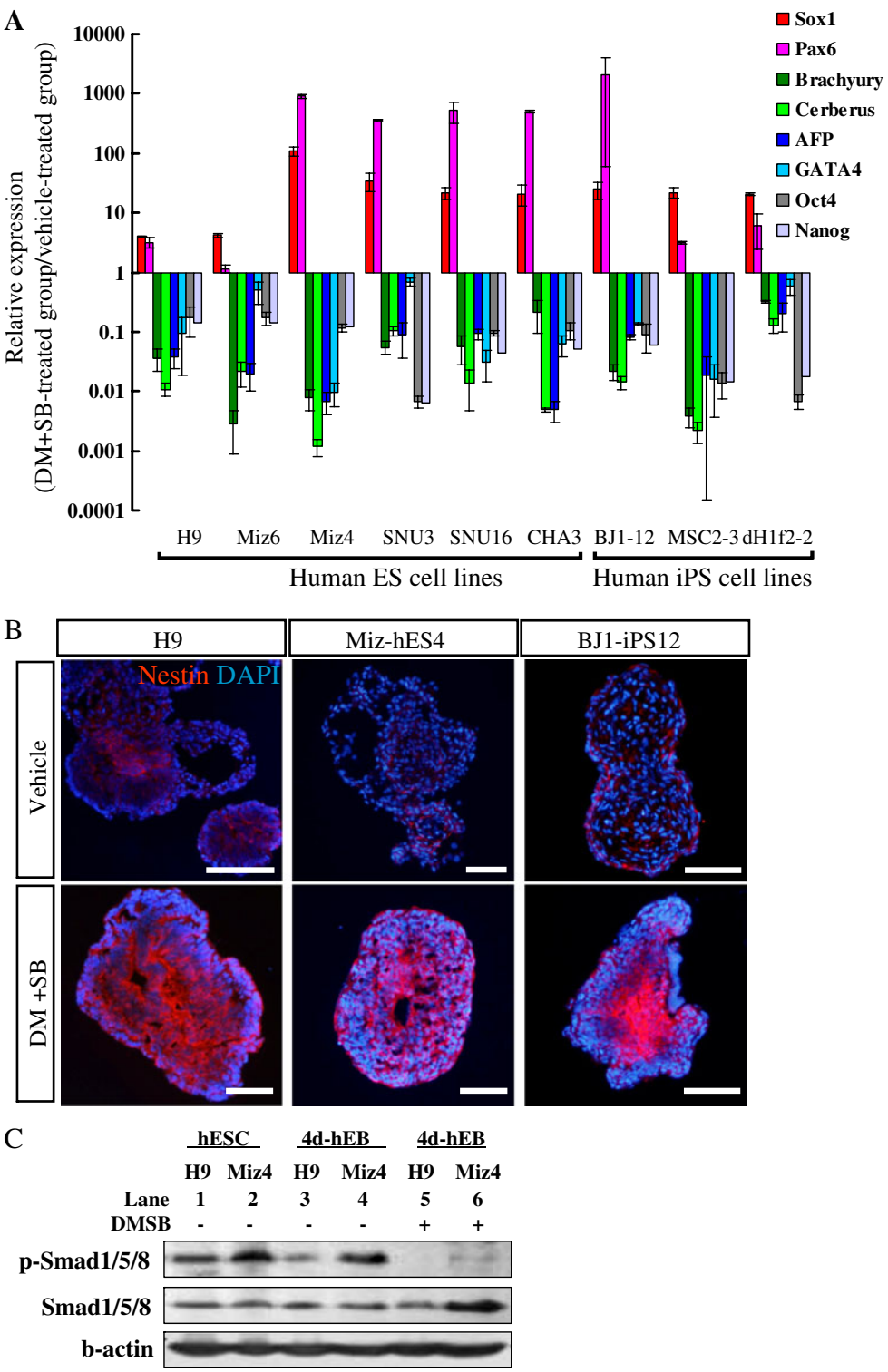

Fig. 5 Inhibition of BMP and Activin/Nodal signaling pathways efficiently overcomes differentiation propensity among human ESC and iPSC lines. **a** Treatment of hPSC lines with both DM and SB431542 dramatically increased the expression of neuroectodermal markers, while significantly reducing that of meso/endodermal and undifferentiation markers. Noticeably, the cell lines that had innate differentiation propensity unfavorable toward neural lineage such as Miz-hES4, SNU-hES3, SNU-hES16, CHA-hES3, and BJ1-iPS12, expressed Sox1 and Pax6, the two representative neural markers, upto approximately 1,000-fold higher after DM and SB431542 treatment. On the other hand, cell lines with high potential to become neural cells, such as H9, Miz6, responded much less significantly to the drug treatment (upto about 6-fold). The y-axis as a log scale shows means ± s.e.m of the relative fold increase of gene expression between small molecule-treated and vehicle-treated cells (arbitrarily designated as 1). **b** EB sections from three hPSC lines (H9, Miz-hES4, and BJ1-iPS12) were immunostained with anti-nestin antibody. **c** Level of phospho-Smad1/5/8 which corresponds to intracellular activity of BMP signaling was analyzed for H9 and Miz-hES4 cells. Samples were prepared in undifferentiation state (lanes 1 and 2) and at 4 days after spontaneous differention (lanes 3, 4, 5 and 6). In some samples, DM and SB431542 were treated during differentiation (lanes 5 and 6). Thirty μg of total proteins were loaded onto each lane of the gel. DM, dorsomorphin; SB, SB431542. Scale bar: 50 μm

Fragmented colonies of the hPSCs were first cultured in suspension in EB medium for 4 days, and subsequently grown on Matrigel-coated dish in N2 medium supplemented with 20 ng/ml bFGF for 6 more days. As expected, H9 and Miz-hES6 cells produced a lot of colonies with NPC-containing rosette structures, while rosette structures were hardly observed in differentiating colonies from Miz-hES4, and BJ1-iPS12 cells (Fig. 6a, b). This observation

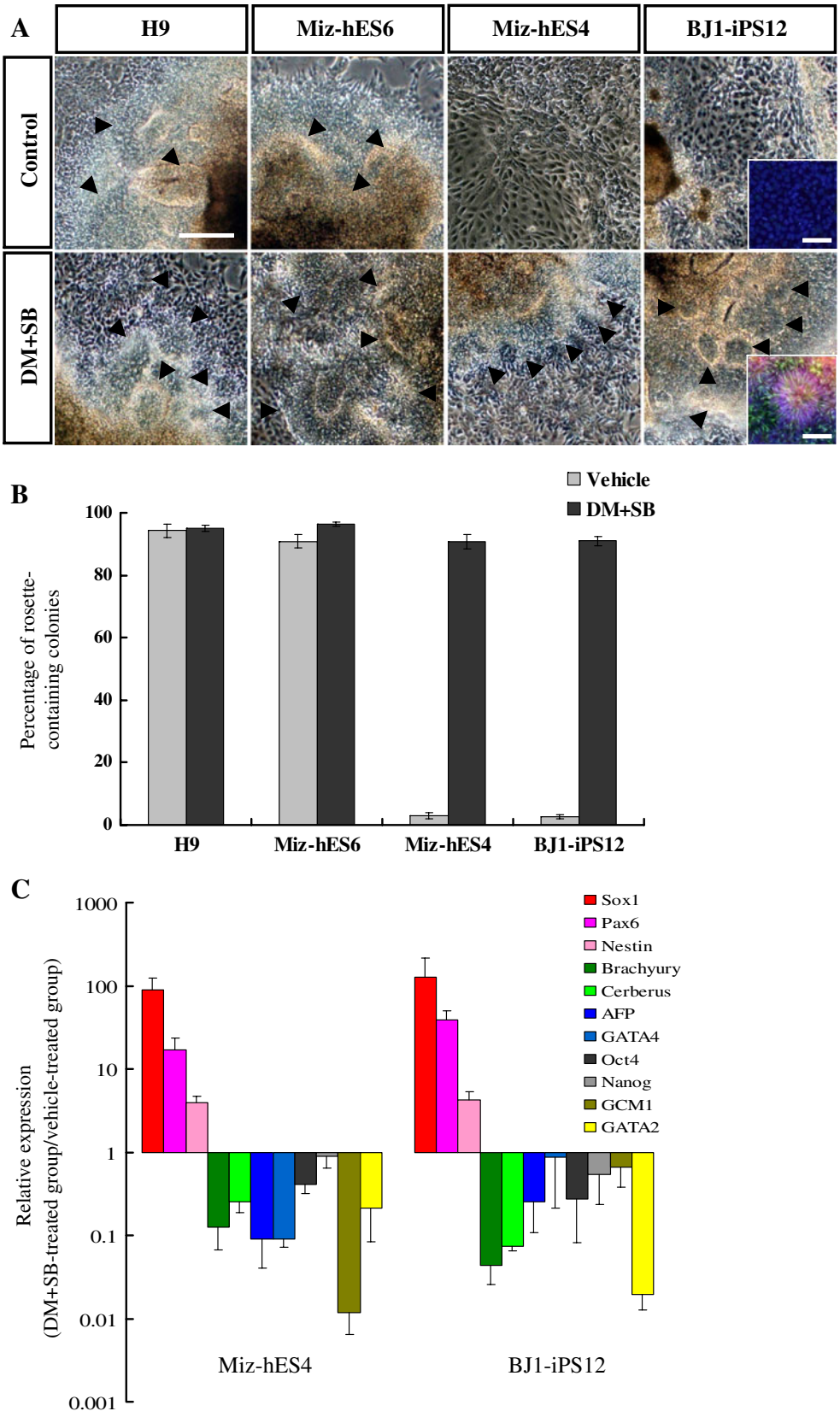

Fig. 6 Inhibition of BMP and Activin/Nodal signaling pathways induces efficient neural cell generation regardless of their innate differentiation propensity after directed differentiation of hPSCs. **a** EBs cultured for 4 days with or without treatment of DM and SB431542 were attached to the culture dishes and grown for 6 more days in neural induction medium (N2 media supplemented with 20 ng/ml of bFGF). The neural rosette structures were evident in some colonies as indicated by arrowheads. *Insets*: Rosettes were stained with both anti-Nestin (*green*) and anti-Sox1 (*red*) antibodies. Scale bar: 20 μm. **b** Percentages of neural rosette-containing colonies from 4 hPSC lines, H9, Miz-hES6, Miz-hES4, and BJ1-iPS12, were examined with or without treatment with DM and SB431542. **c** Effect of DM/SB431542-treatment on the expression level of ectoderm, endoderm, mesoderm, trophoblast, and undifferentiation markers was assessed by qRT-PCR. The relative fold increase of gene expression in DM/SB431542-treated group over vehicle-treated group (arbitrarily designated as 1) are represented on a logarithmic scale. All experiments were repeated at least three times

indicates that formation of neural cells from hPSC lines is also affected by the innate differentiation propensity of the cells even after directed differentiation.

When treated with DM and SB431542 for the first 4 days of suspension culture in EB medium, H9 and Miz-hES6 cells generated slightly higher number of neural rosette-containing colonies (> 90% of total colonies) compared with vehicle-treated samples (Fig. 6a and b). Strikingly, the percentage of neural rosette-containing colonies was increased from about 2% to about 90% when Miz-hES4, and BJ1-iPS12 cells were differentiated in the presence of DM and SB431542 (Fig. 6a and b). qRT-PCR analyses also showed that DM/SB431542-treatment dramatically increased the expression of neuroectodermal markers (Sox1, Pax6 and nestin), whereas reduced the expression of endo/mesodermal (Brachyury, Cerberus, AFP and GATA4), trophoblast (GATA2 and GCM1), and undifferentiated hESC (Oct4 and Nanog) markers in Miz-hES4, and BJ1-iPS12 cells (Fig. 6c). Taken together, these results indicate that innate differentiation propensity seen after spontaneous differention of hPSCs is still evident after directed differentiation and this can be overcome, at least in part, by modulation of intracellular signaling pathways.

Collectively, our results suggested that neural cells are efficiently generated from hESCs and hiPSCs by simultaneous modulation of BMP and Activin/Nodal signaling pathways no matter what spontaneous or directed differentiation procedure was used. This study presents an interesting possibility that hPSC lines with various differentiation propensity can be efficiently coaxed into specific cell types of interest by modulating key signaling pathways involved in the fate determination of the cells.

Discussion

Recent study using hESC lines demonstrated huge differences in differentiation propensity among some lines; after spontaneous differentiation, many three germ layer marker genes were differentially expressed more than 100-fold among different hESC lines [1]. In this study, we also reported significant differences in differentiation propensity among hESC lines. Furthermore, we demonstrated for the first time that hiPSC lines also retained varing innate differentiation potential. The innate differentiation propensity of hPSCs was thought to be attributed to both genetic diversity and diverse epigenetic regulation among different hESC lines [1]. Additionally, we speculate that innate basal activities of intracellular signaling cascades implicated in cell fate determination might be different among hPSC lines with different differentiation propensity. In support of this notion, BMP signaling activity in Miz-hES4 line which has low neural differentiation potential were found to be higher than that in H9 cells (Fig. 5c).

The innate differentiation propensity may pose problems in future patient-specific cell therapy using hiPSCs, let alone hESC-mediated cell therapy, since only a handful hiPSC lines established from a single patient may not possibly contain hiPSC lines with desirable differentiation propensity. Therefore, an efficient method of coaxing hPSCs with different differentiation propensity into specific cell types of interest needs to be established for both basic research and clinical applications of hPSCs. In this study, we investigated whether various hiPSC as well as hESC lines with significantly different differentiation propensity can be efficiently coaxed into neural cells by inhibiting both BMP and Activin/Nodal pathways. BMP pathway is shown to be involved in differentiation into trophoblast [18] or extraembryonic endoderm [19]. Intriguingly, blocking of BMP pathway with noggin was shown to promote differentiation of ESCs into neural lineage [19, 20]. On the other hand, Activin/Nodal signaling pathway is implicated in mesoderm/endoderm specification during early development ("gastrulation" stage) [10, 21]. Paradoxically, this pathway is also known to play an important role in the maintenance of stemness of hESCs [11, 13, 14, 22, 23]. *In vivo* evidence suggests that Activin/Nodal pathways inhibit neuroectoderm formation during early embryo development and precocious neural differentiation occurs when this pathway is malfunctioning [12]. Based on this information, we reasoned that blocking of both BMP and Activin/Nodal pathways would promote neural differentiation of hPSCs. To block BMP or Activin/Nodal pathways, we used small molecules, DM and SB431542, respectively, instead of peptide antagonists described in previous studies [19, 20, 24, 25]. Small molecules are advantageous since they are relatively inexpensive, have higher penetrating capability into cell masses, and tend to be more stable than peptide inhibitors. In fact, we found that DM, a small molecule inhibitor, produced more drastic effect than noggin, a peptide antagonist (Fig. 2b and c).

As expected, our results clearly demonstrated that inhibition of both pathways robustly increased neural cell differentiation, while suppressing the differentiation into the other lineages (Figs. 4, 5, and 6). Interestingly, the fold increase of neural marker expressions between control (vehicle (DMSO)-treated cells) and inhibitor (DM/SB431542)-treated cells were much higher in the cell lines that had innate differentiation inclination unfavorable to neural lineage, such as Miz-hES4, SNU-hES3, SNU-hES16, CHA-hES3, and BJ1-iPS12 cells (Figs. 1 and 5a). This result indicated that the huge difference in differentiation propensity among various hPSCs could be overcome, at least in part, by the inhibition of both BMP and Activin/Nodal pathways, leading to efficient neural formation even from the hPSCs with unfavorable differentiation propensity towards neuroectodermal lineage (i.e.

Miz-hES4 and BJ1-iPS12; Fig. 6a and b). In support of this result, the difference in the level of p-Smad1/5/8, active form of Smad1/5/8, between H9- and Miz-hES4-derived EBs was reduced by the treatment with DM and SB431542 (Fig. 5c).

In summary, we show in this report that simultaneous inhibition of BMP and Activin/Nodal pathways promotes efficient neural differentiation from hiPSCs as well as hESCs, regardless of their innate differentiation propensity. This type of approach would be especially important for future patient-specific cell therapy using hiPSCs, let alone hESC-based cell therapy, since it could allow us to efficiently generate any cell type of interest even from a small number of cell lines established from a single patient.

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