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Effects of bone morphogenetic protein 2 on Id expression and neuroblastoma cell differentiation ☆

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ABSTRACT

Bone morphogenetic proteins (BMPs) are secretory signal molecules that have a variety of regulatory functions during embryonic morphogenesis. BMP2 has been shown to induce differentiation in many cell types, mediated through the activation of its target genes: the inhibitors of differentiation (Id1-3) and key transcription factors. In this study, we investigated the effects of BMP2 on mouse neuroblastoma (Neuro2a) cell differentiation and regulation of the expression of Id1-3 and neural-specific transcription factors. Our results showed that BMP2 stimulation upregulated Id1-3 expression at the early stage of application by involvement of the Smad signaling pathway. BMP2 caused phosphorylation of Smad1/5/8 followed by upregulation of Id1-3. Co-incubation with Noggin, a BMP antagonist, or Smad1 siRNA transfection significantly inhibited phosphorylation of Smad1/5/8 and upregulation of Id protein. Furthermore, our results showed that BMP2-induced differentiation of Neuro2a cells into neurons by downregulating the expression of Id1-3 proteins and upregulating the expression of neural-specific transcriptional factors Dlx2, Brn3a, and NeuroD6. The results suggested that the transient upregulation of Id1-3 expression during the early phase of BMP stimulation may play a role in lineage specification and promote differentiation of neuroblastoma cells towards a neuronal phenotype. Subsequently, a coordinated increase in expression of proneural transcription factors and a decrease in Id1-3 expression may culminate in the transition from proliferation to neurogenesis and the terminal neuronal differentiation of neuroblastoma cells.

1. Introduction

Neuroblastoma is one of the most frequent extracranial solid tumors occurring in infancy and childhood. Neuroblastoma cells show the properties of developing sympathetic neuroblasts. Previous work has shown that all-trans retinoic acid (RA) treatment can induce neuroblastoma cell differentiation (Lopez-Carballo et al., 2002; Jogi et al., 2002), and it is frequently applied in clinics. Recently it was shown that bone morphogenetic protein 2 (BMP2) is capable of inducing growth arrest and neuronal differentiation of rat pheochromocytoma PC12 cells (Iwasaki et al., 1996) and human neuroblastoma-derived cell lines (SH-SY5Y and RTBM1) (Nakamura et al., 2003). However, the signaling pathways that lead to the induction of differentiation of these cells by BMP2 remain poorly understood. In addition, neuroblastoma seems to originate from a disruption of the normal program of neuroblast differentiation and maturation. A clearer understanding of the mechanisms of neuroblast development could thus have relevance for the diagnosis and treatment of these invasive tumors.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily of pleiotropic growth factors and were originally identified for their ability to induce bone and cartilage formation (Wozney, 1992, 1998). Recent studies have shown that BMPs play important roles in regulating morphogenetic processes of various organ systems, including the nervous system, throughout embryonic development (Hogan, 1996). The BMPs have been implicated in the specification of the embryonic dorsal-ventral neural axis (Furuta et al., 1997; Liem et al., 1997; Hebert et al., 2002). Regional expression of levels BMPs have been correlated with both cell proliferation and cell differentiation (Panchision et al., 2001; Liu et al., 2004). BMPs also serve an essential role in initiation of neuronal differentiation (Mehler et al., 2000; Li et al., 1998). The BMP family has more than 20 members, and among them BMP2, BMP4, and BMP7 are expressed mainly in the nervous system.

BMPs signal through binding and heterodimerization of BMP type I and II serine/threonine kinase receptors. BMPs can interact with distinct type I receptors; BMP2 preferentially binds to

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BMPRIa, while BMP4 has a higher affinity for BMPRIb. Activated BMPRI and II heterodimerization leads to the phosphorylation of the receptor-regulated Smads (R-Smads; Smad1, Smad5, and Smad8). The receptor Smads then form complexes with the common mediator Smads (Co-Smad) and are translocated into the nucleus where they regulate gene transcription (ten et al., 2003; Abe, 2006). Smad-binding elements are found on the promoters of inhibitors of differentiation or inhibitors of DNA binding (Id) genes (Lopez-Rovira et al., 2002). Thus BMPs have been shown to regulate Id expression in several cell types (Hollnagel, et al., 1999; Korchynskyi and ten, 2002; Lopez-Rovira et al., 2002; Nakashima et al., 2001).

Recent work has revealed that Id proteins are one of the most important targets of BMPs and that they may be responsible for the biological activities of BMPs. There are four mammalian Id proteins (Id1-4) belonging to the helix-loop-helix (HLH) transcription factor family. As key regulators of tissue-specific gene expression, basic helix-loop-helix (bHLH) transcription factors form homo- or hetero-dimers and bind to the E-boxes (CANNTG) of the DNA sequence through their basic domains to activate the transcription of target genes. Because Id proteins lack this basic domain for DNA binding, heterodimers of Id proteins and bHLH proteins cannot bind to the E-box, and thus act as dominant-negative regulators that interfere with the transcriptional activities of target genes. Id proteins are therefore regarded as inhibitors of cell differentiation and are downregulated during this process. However, recent research has shown that Id proteins also participate in the process of cell differentiation. It is reported that nerve growth factor (NGF) could induce transient increase of Id1-3 gene transcription during the early phase of PC12 cell differentiation (Nagata and Todokoro, 1994). The upregulated expression of Id1-3 in the differentiation of osteoblasts induced by BMP2 (Peng et al., 2004) suggests a role for Id proteins in BMP-induced osteoblast lineage-specific differentiation.

In addition to Id genes, Smad complexes can also recruit transcriptional activators and repressors to regulate target gene expression (Kawabata et al., 1998; Miyazono and Miyazawa, 2002; ten et al., 2003). It is also known that bHLH transcription factors working as intrinsic signals play an important role in determining neuronal cell proliferation, lineage specification and terminal differentiation. During recent years, many other transcription factors besides bHLH transcription factors have been identified to participate in the regulation of neuronal cell differentiation. Transcription factors such as Dlx2 and Brn3a homeodomain factors and NeuroD6 bHLH transcription factor are known to play a role in neuronal lineage commitment and regulation of target gene transcription. This suggests that BMPs can control the cell differentiation process by regulating the expression of Id and other transcription factors. However, the role of BMP and Id protein signaling in regulating neuroblastoma cell differentiation has not been elucidated.

In this study, we investigated BMP2-induced activation of Smad signaling pathway in the regulation of target genes Id1-3 in Neuro2a cells. Furthermore, we examined the effect of BMP2 on the differentiation of mouse neuroblastoma cells. Finally, we sought to determine the possible functional roles of Id proteins in BMP2-induced neuroblastoma cell differentiation.

2. Materials and methods

2.1. Cell culture and treatment

Neuro2a cells purchased from ATCC were cultured in Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA) supplemented with 10% fetal calf serum, 0.2 mM L-glutamine, and antibiotics, in a humidified incubator at 37 °C with 5% CO_2. The medium was changed every other day, and the cells were passaged when they reached confluence. Recombinant human BMP2 (R&D Systems, Inc., Minneapolis, MN, USA), Noggin (R&D systems) and retinoic acid (RA) (Sigma-Aldrich, Inc., St. Louis, MO, USA) were added to the culture medium at the indicated concentrations.

2.2. Cell proliferation and differentiation assays

Neuro2a cells were seeded in a 96-well plate and treated with BMP2 (25 ng/ml) in culture medium. Cells were collected at the indicated time periods and a cell proliferation assay was carried out using a CyQUANT Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA, USA). In this assay, the change in the cell number has a linear relationship with the reading of absorbance of fluorescence at 485 nm. For the differentiation assay, differentiated Neuro2a cells, with neurites whose length was at least double the diameter of the cell body, were counted and expressed as a percentage of the total number of cells.

2.3. RNA extraction and real-time PCR

Cells were treated with Trizol reagent (Gibco, Life Technologies, Grand Island, NY, USA) after PBS wash, and the total RNA was prepared according to the manufacturer's instruction. The cDNA was synthesized by using Superscript III RT Reaction Mix (Invitrogen). Real-time PCR reactions were performed in an iCycle iQ™ Multi-color Real-Time PCR Detection System Instrument (BIO-RAD, Hercules, CA, USA). Gene-specific primer pairs are listed in Table 1. All reactions were performed with SYBR Green qPCR SuperMix (Invitrogen) in triplicate. The comparative threshold cycle method (ΔΔCt) was used to quantify the mRNA expression levels of different genes. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by staining with ethidium bromide.

2.4. Western blot assay

After a PBS wash, cells were extracted in cold RIPA lysis buffer containing Tris–HCl (50 mM, pH 8.0), NaCl (150 mM), Triton X-100 (1%), sodium deoxycholate (0.5%), SDS (0.1%) and protease inhibitor [PMSF (1 mM), leupeptin (1 mg/ml), aprotinin (1 mg/ml)]. The proteins were separated by 12% acrylamide gel elecrophoresis and transferred to a PVDF membrane. After blocking for 1 h in 10% skim milk at room temperature, membranes were incubated overnight at 4 °C with antibodies to Id1-3 (Santa Cruz Biotechnology, Inc. CA, USA), neuron-specific enolase (NSE, Chemicon International Inc., Temecula, CA, USA), pSamd1/5/8 (Cell Signaling Technology, Danvers, MA, USA),

Table 1
Primer sequences.

Gene names	Forward primer	Reverse primer
Id-1	ccagtgggtagagggtttga	agaaatccgagaagcacgaa
Id-2	acccgatgagtctgctctac	ctggttctgtccaggtctct
Id-3	aggtgtctcttttcctccct	atgtcgtccaagaggctaag
GAPDH	accacagtccatgccatcac	tccaccaccctgttgctgta
BMPRIa	cagacttggaccagaagaagcc	acattctattgtcctgcgtagc
BMPRIb	aagaagatgactctggaatgcc	atccacacctcgccatagcg
BMPRII	aggcccaattctctggatct	cactgccattgttgttgacc
Dlx2	atgtctcctactccgccaaa	tggcttcccgttcactattc
Brn3a	gcagcgtgagaaaatgaaca	tttcatccgcttctgcttct
NeuroD6	ctgaggattggcaagagacc	gctgtggtagggtgggtaga
GAP-43	ggctctgctactaccgatgc	catcttcagccttggaggac

Smad1/5/8 (Santa Cruz), pErk and total Erk (Cell signaling Technology) and β-actin (Sigma). The following day, after washing 3 times in TBST (Tris-buffered saline, pH 7.4, 0.1% Tween-20), membranes were incubated for 1 h at room temperature in HRP-conjugated goat anti-rabbit secondary antibody and HRP-conjugated goat anti-mouse secondary antibody (Cell Signaling Technology). Blots were then developed using an enhanced chemiluminescence system (ECL; Amersham Corp., Arlinghton Heights, IL, USA) according to the manufacturer's instructions.

2.5. Id1-promoter luciferase assay

The cells were seeded in a 24-well plate before transfection. The next day, the cells were co-transfected with 0.5 μg Id1-promoter plasmids and 0.01 μg pRL-luc plasmid as an internal control for normalizing transfection efficiency using Fugene 6 transfection reagent (R&D Systems). The pGLbasic3 plasmids were used as an external control. Twenty-four hours after transfection, the cells were rinsed with PBS and treated with BMP2 for additional 24 h. Cells were extracted in passive lysis buffer (Promega Corporation, Madison, WI, USA) and luciferase activity was assayed according to the protocol. The assays were carried out in triplicate.

2.6. Immunocytochemistry

Cells were cultured on poly-L-lysine (25 μg/ml, Sigma) coated coverslips and treated with 25 ng/ml BMP2 for different time periods. After washing 3 times in PBS, cells were fixed in cold methanol for 30 min, and blocked in 10% normal goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 in 0.01 M PBS for 1 h at room temperature. The cells were then incubated with anti-Smad1/5/8 antibody (Cell Signaling Technology), and BMPRIb, BMPRIa, and BMPRII antibodies (Santa Cruz) in the blocking buffer overnight at 4 °C. On the following day, the slides were washed in PBS (3×15 min) and incubated with donkey anti-rabbit 488 secondary antibody and donkey anti-goat 568 (1:400 dilution, Molecular Probes, Eugene, OR, USA) in PBS for 1 h at room temperature. Cell nuclei were counterstained with DAPI. On the negative controls the primary antibodies were omitted.

2.7. siRNA transfection

Smad1 siRNA and control siRNA were purchased from Dharmacon (Chicago, IL, USA), and the transfection was performed using lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were seeded 1 day before transfection at 80–90% confluence, without antibiotic. The siRNA and lipofectamine 2000 were diluted in Opti-MEM separately for 5 min, mixed for 20 min, and then added to the medium. The cells were collected 48 h after transfection. The downregulation efficiency of Smad1 siRNA was tested by western blot.

2.8. Statistical analysis

Data are presented as the average from three independent experiments. Statistical analyses were performed using the software PRISM 4 (Prism v4.0, Graphpad Software, Inc., San Diego, CA, USA). One-way analysis of variance and Turkey multiple comparisons test or Student's *t*-test was used to determine the statistical significance of differences. Asterisks in the figures denote a statistically significant difference compared with the corresponding groups ($^{*}P < 0.05$; $^{**}P < 0.01$). Error bars represent standard error of the mean.

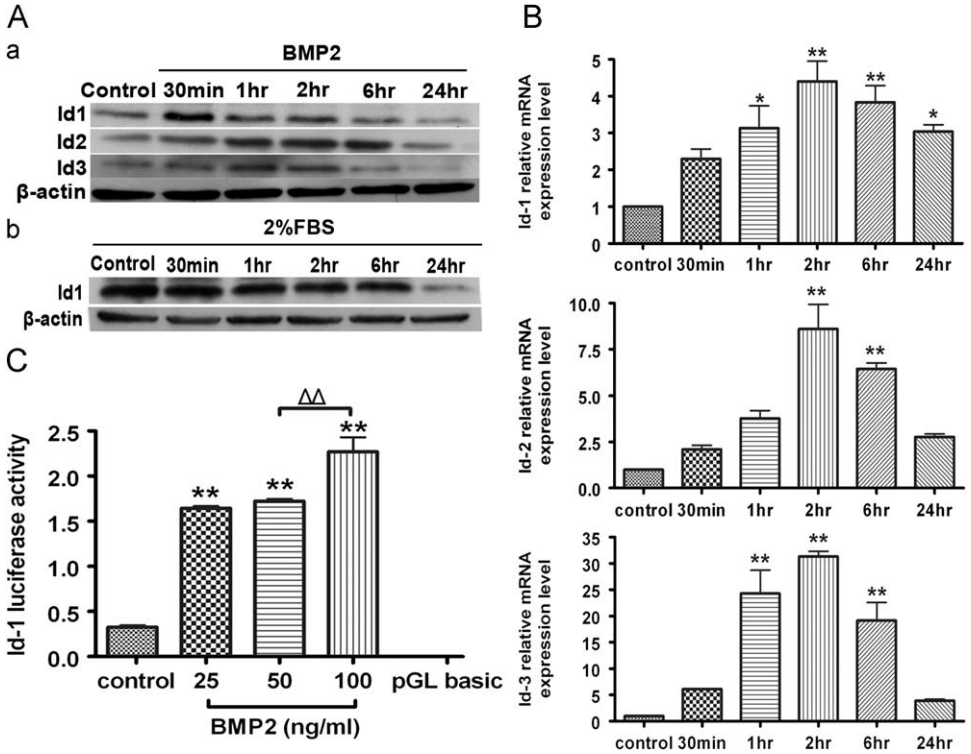

Fig. 1. BMP2 upregulates Id1-3 expression in Neuro2a cells. (Aa) Western blot result showed that addition of BMP2 was followed by a significant increase in Id1-3 protein levels in Neuro2a cells as early as 30 min, and returned to a basal level after 24 h of stimulation. (Ab) There was no upregulation of Id1 expression in the untreated group at the corresponding time courses. (B) Real-time PCR results showed that Id1-3 mRNA expression was significantly increased after treatment with BMP2. (C) Id1 promoter activity was increased by BMP2 in a dose-dependent manner.

3. Results

3.1. Effect of BMP2 stimulation on Id1-3 expression levels in Neuro2a cells

BMP2 treatment of Neuro2a cells led to a significant increase in Id1-3 protein levels, as early as 30 min after the addition of BMP2 (Fig. 1Aa), while there was no increase in Id1 protein expression level in untreated cells (Fig. 1Ab). Analysis of Id1-3 gene expression revealed that treatment of Neuro2a cells with BMP2 resulted in a considerable increase in Id1-3 mRNA levels (Fig. 1B). Although the upregulation of Id1-3 protein and mRNA levels by BMP2 represented a transient response that returned to basal levels after 24 h of stimulation, the kinetics of induction and the magnitudes of expression were highly individual between different Ids. The reporter assay indicated that application of BMP2 at 25 and 50 ng/ml already led to a four-fold increase of Id1-promoter activity compared with the control group. Id1-promoter activity was increased six-fold in response to 100 ng/ml of BMP2 (Fig. 1C).

3.2. Id1-3 activation by BMP2 is through phosphorylation of Smad1/5/8 proteins in Neuro2a cells

The BMP receptors BMPRIa (Fig. 2Aa, c), BMPRIb (Fig. 2Ad, f) and BMPRII (Fig. 2Ag, i) were expressed in Neuro2a cells. Cell nuclei counterstained with DAPI were seen (Fig. 2Ab, e, h). RT-PCR results showed that mRNA for BMP type I and type II receptors were detected in Neuro2a cells (Fig. 2B).

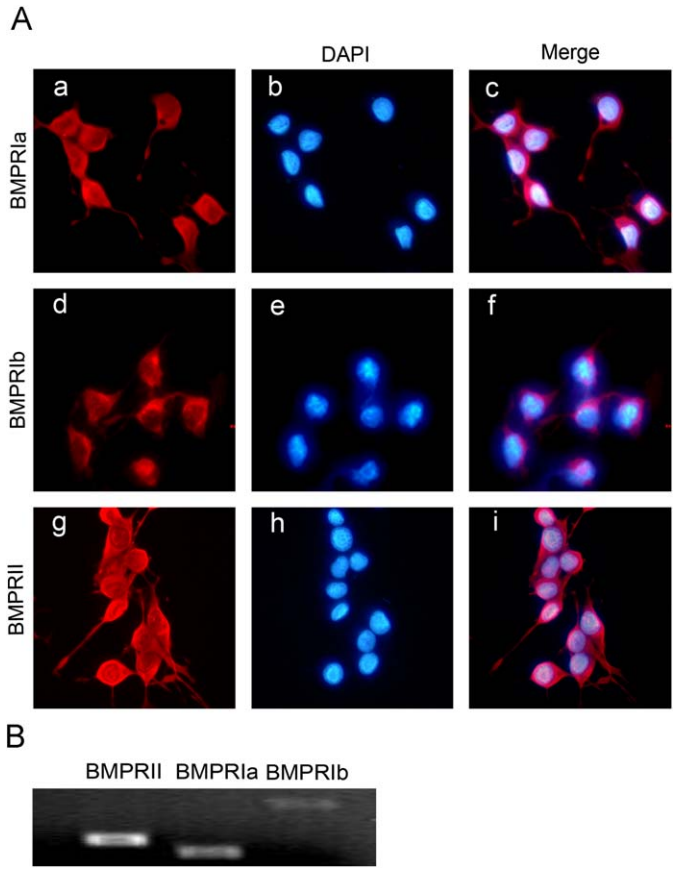

Fig. 2. Expression of BMP receptors in Neuro2a cells. (A) Immunofluorescence micrographs show representative views of Neuro2a cells expressing BMPRIa (Aa), BMPRIb (Ad) and BMPRII (Ag). Nuclei were counterstained with DAPI (Ab, e, h). (B) RT-PCR analysis detected expression of BMPRIa, BMPRIb and BMPRII mRNA in Neuro2a cells.

pSmad1/5/8 proteins were detected in BMP2-treated Neuro2a cells (Fig. 3A) at 30 min after the addition of BMP2 and remained at a high level between 1 and 6 h, whereas pSmad1/5/8 proteins were not detected in untreated control cells. In treated cells, the level of pSmad1/5/8 proteins decreased 24 h after BMP2 application. There was no change in the total Smad1/5/8 protein levels after the BMP2 treatment (Fig. 3A). The western blot result was verified by immunocytochemistry staining for expression of pSmad1/5/8, which showed that although pSmad1/5/8 expression was not detected in control cells (Fig. 3Ba, c), high levels of pSmad1/5/8 proteins were present in the nuclei of these cells 30 min after BMP2 treatment (Fig. 3Bd, f). Knockdown of Smad1 using siRNA abolished the effect of BMP2 on the phosphorylation of Smad1/5/8 proteins and the upregulation of Id1 expression in Neuro2a cells in comparison with control siRNA (Fig. 3C). Similarly to the effects of Smad1 siRNA on Smad protein phosphorylation and Id protein upregulation, transfection of Neuro2a cells with Smad1 siRNA also significantly decreased Id1-promotor activity (Fig. 3D) and Id1 mRNA expression (Fig. 3E) induced by BMP2.

3.3. BMP2 induces Neuro2a cell differentiation

After 6 days in culture, BMP2-treated Neuro2a cells progressively developed into cells compatible with neuronal-like morphology, characterized by neurite outgrowth (Fig. 4Ab), compared with cells left untreated (Fig. 4Aa). Similar patterns were observed with induction of cell differentiation using 10 nM RA (the positive control) (Fig. 4Ad). A significant decrease in cell proliferation was seen after 3 days of BMP2 treatment (Fig. 4B); concomitantly, there was a corresponding increase in the proportion of differentiated cells (Fig. 4C). Western blot analysis showed that Id1-3 protein expression levels were gradually decreased, while the expression of neuronal markers, NSE and phosphorylated extracellular signal-regulated kinase (Erk), progressively increased up to 6 days of BMP2 treatment (Fig. 4D). Furthermore, we confirmed that differentiated Neuro2a cells expressed no detectable Id1 protein 6 days after treatment with RA (Fig. 4E). In real-time PCR analyses, BMP2 increased GAP-43 expression after 2 days of treatment (Fig. 4F). Moreover, the BMP2-induced increase in GAP-43 expression correlated with an increase in the number of cells with neurite outgrowth and the formation of longer neurites (Fig. 4Ab).

3.4. BMP2-differentiated Neuro2a cells express neural-specific transcriptional control genes

Real-time PCR performed on total RNA from Neuro2a cells that were left untreated or treated with BMP2 for 1, 2, or 4 days showed that NeuroD6, Dlx2, and Brn3a mRNA messages were present in untreated cells and increased two- to three-fold with BMP2 treatment (Fig. 5A–C). This effect coincided with the morphological changes that were seen in the Neuro2a cells (Fig. 4Ab) and the decreased Id1-3 protein levels (Fig. 4D) when the cells had undergone differentiation induced by BMP2.

3.5. Noggin blocks the effects of BMP2 on the activation of Smad1/5/8 and Id1 induction

Immunoblots showed that Neuro2a cells pretreated with 200 ng/ml Noggin, a BMP antagonist, demonstrated an absence of pSmad1/5/8 expression and Id1 induction, compared with cells exposed to BMP2 only (Fig. 6A). Immunofluorescence staining

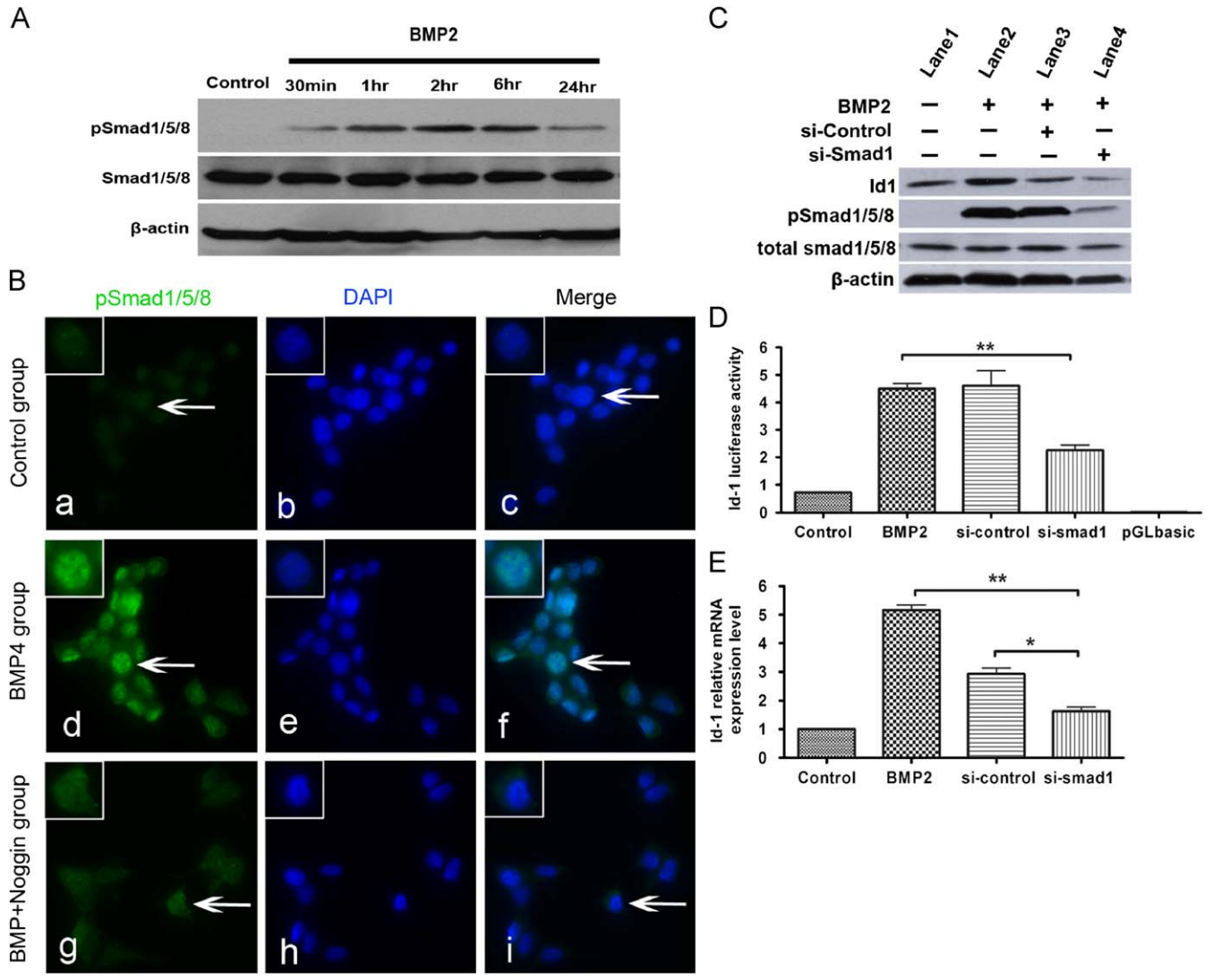

Fig. 3. BMP2 induces Id1-3 expression by phosphorylation and nuclear translocation of Smad1/5/8 in Neuro2a cells. (A) Western blot analysis of pSmad1/5/8 protein expression levels in Neuro2a cells following treatment with 25 ng/ml BMP2 at the indicated time points. BMP2 stimulation increased pSmad1/5/8 protein expression as early as 30 min after the addition of BMP2 and decreased after 24 h in culture, but the expression of total Smad1/5/8 proteins remained the same during the whole period of BMP2 treatment. (B) Cellular location of pSmad1/5/8 proteins was analyzed using immunofluorescence staining. There was no pSmad1/5/8 expression in either the BMP2-untreated (Ba, c) or Noggin-pretreated cells (Bg, i). pSmad1/5/8 immunofluorescence can, however, be detected in the nucleus 30 min after BMP2 treatment (Bd, f). (C) Phosphorylation of Smad1/5/8 proteins and upregulation of Id1 protein expression were inhibited by Smad siRNAs in Neuro2a cells. Cells transfected with Smad1 siRNA prior to stimulation with BMP2 (25 ng/ml) had significantly reduced pSmad1/5/8 protein levels and Id1 protein expression after the addition of BMP2. Smad1 siRNA also reduced Id1 promoter activity (D) and Id1 mRNA expression level (E) in comparison with untransfected or control siRNA transfected cells.

demonstrated that Neuro2a cells do not express pSmad1/5/8 when exposed to Noggin pretreatment (Fig. 3Bg, i). As shown in Fig. 4Ac, neurite outgrowth was not observed in Noggin-pretreated cells stimulated with BMP2. Moreover, Noggin blocked the activation of Id1-promoter activity (Fig. 6B) induced by BMP2.

4. Discussion

BMP2 and BMP4 have been demonstrated to induce Id expression in osteoblast-like cells, embryonic stem cells, marrow stromal cells, epithelial cells and cancer cells (Hollnagel et al., 1999; Ogata et al., 1993; Locklin et al., 2001; Kowanetz et al., 2004; Clement et al., 2000). Id1 has been shown to be an important target of BMPs, and BMPs can increase Id1-promoter activity (Korchynskyi and ten, 2002). However, it is still unknown whether Id1-3 genes are also regulated by BMP2 in Neuro2a cells and whether they are involved in the differentiation of neuroblastoma cells induced by BMP2. In this study, we found that BMP2 can regulate Id1-3 expression and that the effect is time-dependent. Id1-3 mRNA and protein expression levels and Id1-promoter activity were upregulated during the first 24 h after the addition of BMP2, but rapidly decreased below the control level at the initiation of neuroblastoma cell differentiation. The role of BMP2 in neuroblastoma cell differentiation is less clear, but it is possible, on the basis of the correlation between Id expression and transduction of BMP signaling, that BMP2 acts upstream of Ids and that these signaling molecules are required for neuroblastoma cell differentiation.

Id genes are widely distributed in the developing nervous system in a complex and dynamic pattern, and they are implicated in a number of cellular processes, including cell proliferation and differentiation (Andres-Barquin et al., 2000). Ids are responsible

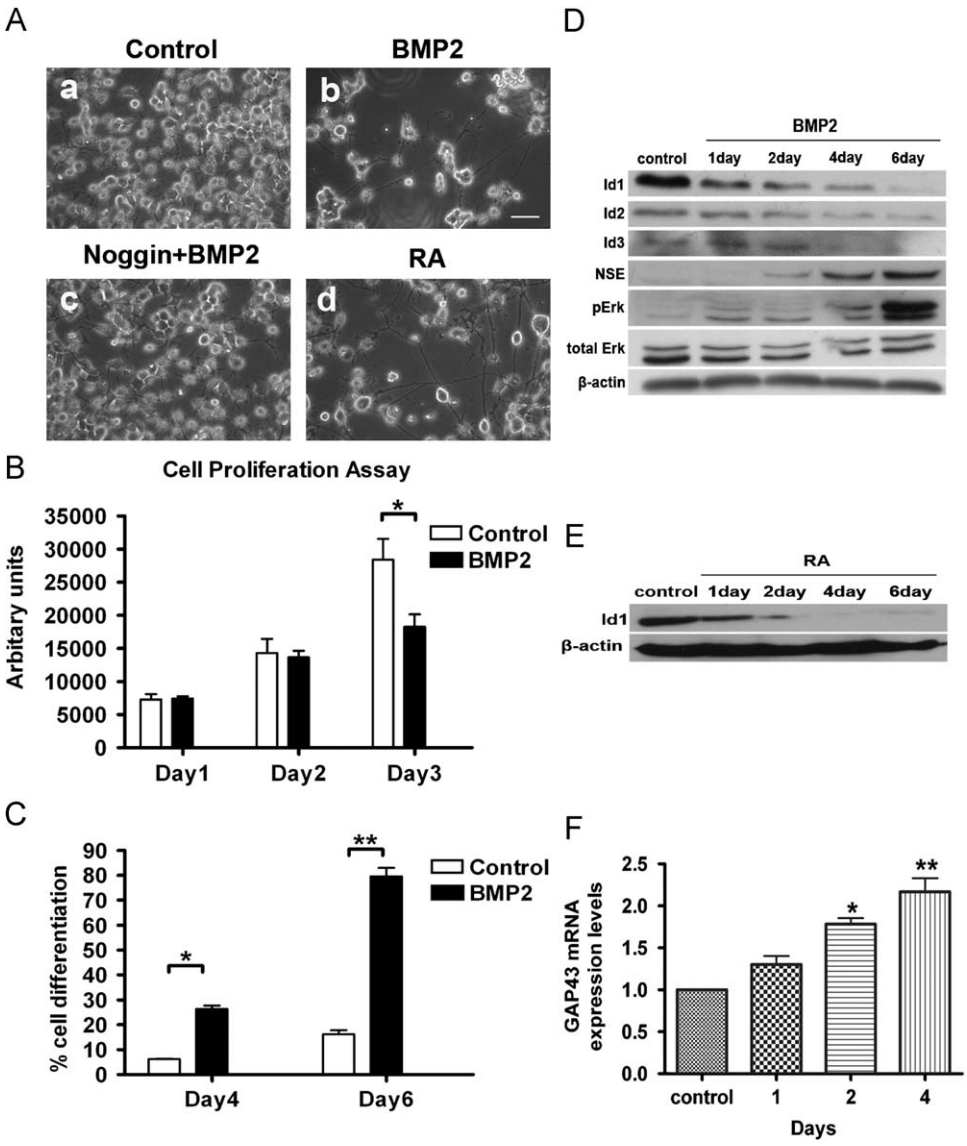

Fig. 4. Induction of neuronal differentiation by BMP2 in Neuro2a cells. (A) Morphological changes induced by BMP2-treated and RA-treated cells were assessed using phase-contrast microscopy. Extensive neurite outgrowth was observed in cells stimulated with either 25 ng/ml BMP2 (Ab) or 10 nM RA (Ad) compared with control (Aa). Conversely, Noggin pretreatment inhibited the effect of BMP2 on cell differentiatioin (Ac). Scale bar: 30 μm. (B) Cell proliferation assay results showed a significant decrease in the percentage of proliferating cells 3 days after BMP2 treatment. (C) Conversely, the percentage of differentiated cells was significantly increased 4 days following BMP2 treatment compared with untreated controls. (D) BMP2 decreased Id1, Id2, and Id3 protein expression and increased expression of the neuronal markers neuron-specific enolase (NSE) and phosphorylated Erk 6 days after treatment. (E) Differentiated Neuro2a cells expressed no detectable Id1 protein 6 days after treatment with RA. (F) BMP2 stimulation also increased the expression of growth-associated protein (GAP)-43 mRNA, a gene associated with neuronal differentiation and neurite outgrowth, as measured by real-time PCR.

for mediating the biological activities of BMPs, and the effects of BMPs on the expression of Ids require BMP/Smad signaling (Langenfeld et al., 2006; Piccirillo et al., 2006). In agreement with these reports, our results showed an enhanced phosphorylation of Smad1/5/8 proteins and nuclear accumulation of phosphorylated Smad1/5/8 proteins in neuroblastoma cells after BMP2 stimulation, suggesting that activation of BMP signaling and translocation of phosphorylated Smad proteins into the nucleus induce activation of target genes such as Id genes. Furthermore, Id1 mRNA and protein expression levels and Id1-promoter activity were reduced when BMP2 signaling was silenced by Smad1 siRNA or treatment with Noggin, a BMP antagonist. Taken together, these data suggested that the ability of BMP2 to induce Smad1/5/8 activation is consistent with Id genes being targets of BMP/Smad signaling in Neuro2a cells. Furthermore, our results suggested that inhibiting BMP signaling with Noggin is sufficient to inhibit the effects of BMP2 on phosphorylation of Smad1/5/8 proteins and the upregulation of Id expression.

We found transient upregulation of Id proteins in the early phase of neuroblastoma cell differentiation. This phenomenon seems paradoxical because Id genes are usually upregulated in proliferating and undifferentiated cells, and downregulated in differentiated cells (Norton et al., 1998). However, the transient increase of Id gene expression before cell differentiation has also been identified in other types of cells. It has been found that Id1-3 function as immediate-early responsive gene products after the stimulation of nerve growth factor (NGF) in differentiating PC12 cells, and it was suggested that upregulation of Id genes may play an important role during the early stage of neural differentiation (Nagata and Todokoro, 1994). Another study showed that BMP2,

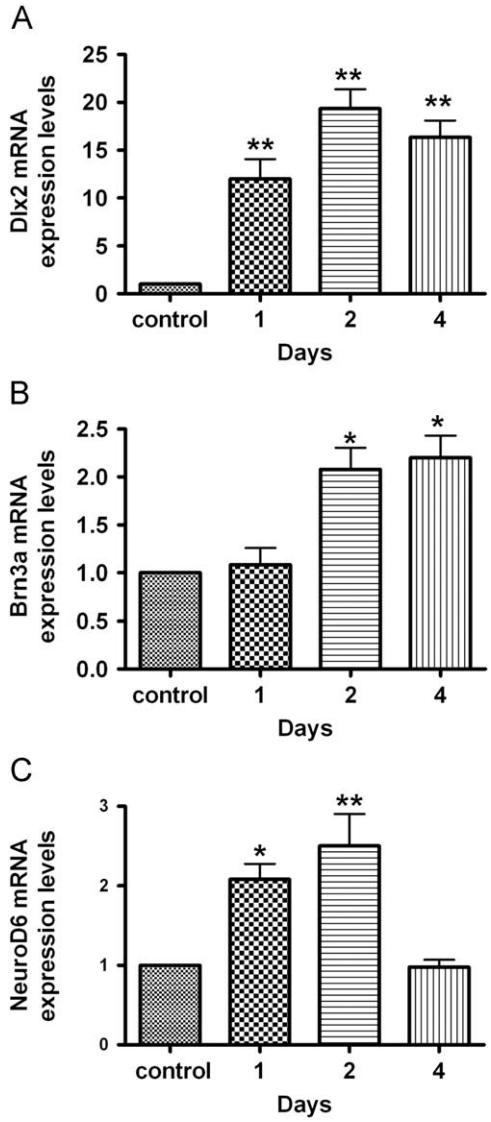

Fig. 5. BMP2 induces neural-specific transcriptional gene Dlx2, Brn3a and NeuroD6 mRNA expressions in Neuro2a cells. Neuro2a cells were cultured with the addition of BMP2 (25 ng/ml) for 1, 2 and 4 days. Total RNA was extracted and expression of Dlx2, Brn3a and NeuroD6 mRNA was quantified by real-time PCR. The relative expression levels of Dlx2 (A), Brn3a (B) and NeuroD6 (C) at the indicated time periods are shown.

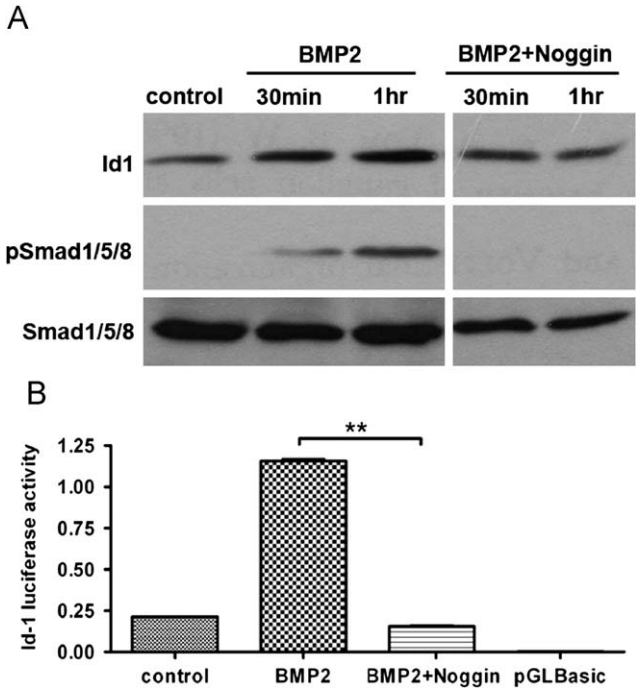

Fig. 6. Noggin inhibits BMP2-induced Id1 expression mediated by Smads in Neuro2a cells. (A) Noggin decreased pSmad1/5/8 protein expression induced by BMP2. Cells pretreated with Noggin (200 ng/ml) showed no detectable pSmad1/5/8 protein expression and Id1 upregulation compared with the group treated with BMP2 (25 ng/ml) alone. (B) Luciferase assay result showed that there was no upregulation of Id1 promoter activity after Noggin pretreatment.

as a potent inducer of differentiation of osteoblasts, can also enhance the expression of Id genes in the early phase of differentiation of osteoblast-like cells, which indicates that the upregulation of Id expression may also play a role in promoting osteoblast differentiation (Ogata et al., 1993). Expression levels of Id1-3 genes were greatly induced during the early stage of BMP9 stimulation and then returned to basal levels 3 days later, suggesting that a balanced regulation of Id expression is critical for BMP-induced osteoblast lineage-specific differentiation of mesenchymal stem cells (Peng et al., 2004). The mechanism by which transient increase of Id expression promotes the initiation of neuroblastoma cell differentiation is intriguing. It can be speculated that Id genes may inhibit a specific set of bHLH transcription factors that may normally inhibit cell differentiation. It is likely that Ids may play a role as a molecular switch for lineage specification through functionally blocking the developmental programs regulated by specific transcription factors (Hollnagel et al., 1999).

In our study, the data indicated that long-term BMP2 treatment of Neuro2a cells resulted in a significant decrease in cell proliferation. At the same time, BMP2 treatment increased the number of differentiated neuroblastoma cells, as demonstrated by the observation of cells exhibiting neuronal morphology, with neurite outgrowth, and expressing neuronal markers such as NSE, phosphorylated Erk and GAP43. Erk 1 and 2 are established neuronal differentiation mediators, for example in PC12 cells (Evangelopoulos et al., 2005). These results indicated that neuroblastoma cells differentiated into neurons when exposed to BMP2. The possible mechanism is that the decreased expression of Id proteins after long-term BMP2 treatment released the inhibitory effects of Id proteins on bHLH poteins and led to the binding of bHLH proteins to E-box to form functional dimmers, thus allowing transcription of cell-specific genes for cell differentiation (Melnikova and Christy, 1996). The suggestion that downregulation of Id expression is a prerequisite for Neuro2a cell differentiation is in line with previous findings that the Id1-3 mRNA transcripts were downregulated during RA-induced differentiation of SH-SY5Y cells, underscoring the role of Id genes in differentiation (Lopez-Carballo et al., 2002; Jogi et al., 2002).

Our results suggested that Id proteins may play a dual role in BMP2-induced neuroblastoma cell differentiation. During the early stage of BMP2 stimulation, the transient upregulation of Id genes may function as a molecular switch directing the transition of uncommitted neuroblastoma cells to the neuronal cell lineage. During the later stage of BMP2 treatment, Id1-3 expression was gradually downregulated coincident with the upregulation of neural transcription factors, which can activate the genes that induce the terminal differentiation of neuroblastoma cells.

A large number of transcription factors have been identified as key determinants of cell fate that regulate the molecular identity of neurons, many of which are members of the bHLH and

homeodomain transcription factor classes (Anderson, 1999; Shirasaki and Pfaff, 2002). Previous work showed that BMP2 can regulate a set of transcription factors, which are involved in regulating neuronal cell differentiation, but it is not clear whether these neural-specific bHLH and homeodomain transcription factors also play a role in mediating downstream effects of BMP signaling in neuroblastoma cell differentiation. One of these transcriptional control factors, Dlx, is a homeodomain transcription factor known to be induced by BMP2 and, in turn, induce expression of genes associated with neuronal differentiation. Studies in a clonal chondroblastic cell line demonstrate that Dlx2 is a downstream target of the BMP2 signaling pathway, and is involved in chondroblast differenation (Xu et al., 2001). It has been reported that Dlx2 was upregulated immediately after the addition of BMP2 in human marrow stromal cells (Locklin et al., 2001). Members of the vertebrate Distal-less (Dlx) family genes are also known to be implicated in neurogenesis during the development of the vertebrate central nervous system (Anderson et al., 2001). Dlx1 and Dlx2 are known to determine the terminal differentiation condition of late-born retinal ganglion cell progenitors (de et al., 2005). In our study, we found that Dlx2 was significantly upregulated 1 day after treatment with BMP2, which indicated that Dlx2 may be involved in regulating Neuro2a cell differentiation induced by BMP2.

Another gene showing increased transcription in response to BMP2 is NeuroD6 (Nex1/MATH-2). The NeuroD6 gene belongs to the NeuroD family – a member of the bHLH superfamily, which is involved in neuronal differentiation. It has been shown that NeuroD6 expression parallels that of the GAP-43 gene during the neurite outgrowth and neuronal differentiation of PC12 cells. NeuroD6 works as a crucial activator of the GAP-43 gene (Uittenbogaard et al., 2003; Uittenbogaard and Chiaramello, 2002). GAP-43 is located mainly in the growth cones and elongating axons of developing neurons (Benowitz and Routtenberg, 1997), and it has been demonstrated changes in GAP-43 expression could be a valuable indicator of PC12 cell differentiation (Das et al., 2004). During neuronal cell differentiation, GAP-43 controls the timing of when proliferating neuroblasts differentiate into post-mitotic neurons (Mani et al., 2000, 2001). Consistent with these observations, our results showed that treatment with BMP2 for 1 day upregulates the NeuroD6 mRNA expression level in Neuro2a cells, and that GAP-43 mRNA expression was upregulated 2 days after the addition of BMP2. Our results suggest that NeuroD6 may activate the GAP-43 gene promoter and induce Neuro2a cell differentiation.

In our study, we found that BMP2 also markedly increased expression of Brn3a, a transcription factor of the POU-domain family, and plays important roles in neuronal cell proliferation and differentiation. Brn3 genes are expressed in distinct but overlapping patterns in the developing retina, and promote differentiation of retinal ganglion cells (Pan et al., 2005; Hutcheson and Vetter, 2001; Liu et al., 2000, 2001). The expression of Brn3a is also upregulated during differentiation of the ND7 cell line derived from sensory neurons (Budhram-Mahadeo et al., 1994; Lillycrop et al., 1992).

In conclusion, our results show that BMP2 signaling could influence Neuro2a cell differentiation by regulating the expression of Id proteins. In addition, we have demonstrated that the bHLH transcription factor NeuroD6 and the homeobox genes Dlx2 and Brn3a are also downstream targets of the BMP2 signaling pathway. Thus, we propose that Ids may function through selective dimerization with stage- and cell-specific transcription factors in the differentiation of Neuro2a cells. The transcriptional actions of the Ids and the significance of the interplay between Ids and transcription factors in neuroblastoma cell differentiation require further investigation.

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