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Accumulation of $p27^{KIP1}$ is associated with BMP2-induced growth arrest and neuronal differentiation of human neuroblastoma-derived cell lines

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Abstract

Bone morphogenetic proteins (BMPs) play an essential role in cell fate determination. In this study, we found that BMP2 treatment resulted in growth arrest and differentiation in human neuroblastoma-derived cell lines, SH-SY5Y and RTBM1. Within 30 min of BMP2 exposure, phosphorylation of Smad1/5 was observed in these cell lines. In RTBM1 cells, BMP2-induced differentiation was accompanied by a significant decrease in the expression level of *DAN*, an antagonist of BMP in frog embryos. Immunoblot analysis revealed that BMP2 treatment caused a down-regulation of p53 family members and hence of cyclin-dependent kinase inhibitor $p21^{WAF1}$. We found a significant accumulation of $p27^{KIP1}$ in response to BMP2, whereas the expression level of Skp2, which is required for ubiquitin-dependent $p27^{KIP1}$ degradation, was decreased during this differentiation process. Our results suggest that $p27^{KIP1}$ contributes to the BMP-induced growth arrest and neuronal differentiation of neuroblastoma, and BMP treatment might provide a new therapeutic strategy.

Keywords: BMP2; DAN; Neuroblastoma; Neuronal differentiation; p53; p73; $p27^{KIP1}$

Neuroblastoma (NBL) is one of the most frequent pediatric solid tumors which is derived from embryonal neural crest cells, and is clinically and biologically heterogeneous. Tumors found in patients under the age of 1 year are usually favorable and often show spontaneous differentiation or regression, whereas tumors in patients identified over the age of 1 year display clinically aggressive behavior [1,2]. Accumulating evidence strongly suggests that favorable neuroblastomas highly express TrkA, a high affinity receptor for nerve growth factor (NGF), while the expression level of TrkA is significantly reduced in advanced neuroblastomas with *MYCN* amplification [3–5]. As expected, the addition of NGF into the primary culture of neuroblastomas expressing TrkA at high level resulted in the promotion of neuronal differentiation, however, most neuroblastoma-derived cell lines did not respond to NGF treatment due to the defects in NGF signaling pathway [6,7]. In an effort to search for additional molecule(s) capable of inducing neuroblastoma cell differentiation, numerous molecules were tested for their ability to promote differentiation. Among them, *all-trans* retinoic acid (RA) was able to induce growth arrest and differentiation of certain neuroblastoma cell lines [8,9]. Gaetano et al. [10] found that RA treatment resulted in the down-regulation of $p34^{cdc2}$ level as well as the induction of the dephosphorylation of pRB in neuroblastoma cells. Recently, it has been demonstrated that the cyclin-dependent kinase inhibitor $p27^{KIP1}$ was accumulated during RA-induced growth arrest and differentiation of neuroblastoma cells [11,12]. Intriguingly, the expression level of p73, a newly identified p53 family member, was significantly increased during RA-induced neuroblastoma differentiation, and ectopically

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expressed p73 was sufficient to induce neuroblastoma differentiation [13].

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGF-β) superfamily, have been implicated in bone formation and bone cell differentiation [14–16]. Like pattern-inducing gene products, Noggin and Chordin, BMP signaling is inhibited by the DAN family members in *Xenopus* embryos [17,18]. Apart from their well-described effects on bone formation, BMPs contribute to the regulation of a variety of biological processes including neuronal differentiation. Iwasaki et al. [19] found that BMP2 possesses an ability to induce neuronal differentiation of rat pheochromocytoma PC12 cells, however, it is not known whether BMPs have an ability to promote neuronal differentiation in human neuroblastoma-derived cell lines. In the present study, we found that BMP2 treatment of human neuroblastoma SH-SY5Y and RTBM1 cells delayed cell growth and induced neuroblastoma cell differentiation. Of note, we found a significant accumulation of $p27^{KIP1}$ in response to BMP2 during this differentiation process.

Materials and methods

Cell culture. SH-SY5Y and RTBM1 cells derived from human neuroblastoma were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were maintained under an atmosphere of 5% CO_2 at 37 °C. For neurite extension assays, RTBM1 or SH-SY5Y cells were treated with recombinant human BMP2 at a final concentration of 1 nM (Yamanouchi Pharmaceutical) and neurite outgrowth was allowed to proceed for 6 days.

Cell proliferation. To evaluate cell proliferation, SH-SY5Y and RTBM1 cells were plated onto 12-well cell culture dishes at a density of 1×10^4 and 5×10^4 cells/well in 1 ml of culture medium containing 10% FBS, respectively. Cells were allowed to adhere to the bottom of the cell culture dish for 24 h. At the indicated time periods, cells were trypsinized and cell counting was carried out in triplicate using a Coulter Counter (Coulter Electronics).

BrdU incorporation during DNA synthesis. After exposure to BMP2 (at a final concentration of 1 nM), cells were treated with 10 μM of 5-bromo-2′-deoxyuridine (BrdU; Roche Molecular Biochemicals) for 1 h. Cells were then washed three times with ice-cold phosphate-buffered saline (PBS) and fixed with 70% ethanol for 20 min at −20 °C. After washing with PBS, cells were incubated with a monoclonal anti-BrdU antibody diluted 1:10 in the incubation buffer (66 mM Tris–Cl, pH 7.5, 0.66 mM $MgCl_2$, and 1 mM β-mercaptoethanol) for 30 min at 37 °C, washed three times with PBS, incubated with an FITC-conjugated goat anti-mouse IgG secondary antibody diluted 1:10 in PBS for 30 min at 37 °C, and again washed three times with PBS. Cell nuclei were stained with PI. The stained cells were visualized under a confocal laser scanning microscope (Olympus).

RNA preparation and RT-PCR analysis. Total RNA was prepared from cultured cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For RT-PCR analysis, 1 μg of total RNA was reverse transcribed with SuperScript II (Life Technologies) primed with random primers at 42 °C for 2 h, followed by 15-min denaturation at 65 °C and then quick cooling. The resulting cDNA was subjected to the PCR-based amplification with the following oligonucleotides: for human *BMPR-IA*, 5′-GCAATTGCTCATCGAGAC C-3′ and 5′-CGAAGGTGTAGATGTCAGCC-3′; for human *BMPR-IB*, 5′-TCTTCACCACAGAGGAAGCC-3′ and 5′-AAGCCACTGAC AGAAGAGTAGG-3′; for human *BMPR- II*, 5′-AATGCAGCCATA AGCGAGG-3′ and 5′-TGGTACTCTGGTACGGATTCC-3′; for human $p27^{KIP1}$, 5′-ATGTCAAACGTGCGAGTGTC-3′ and 5′-CTCT GCAGTGCTTCTCCAAG-3′; and for human *GAPDH*, 5′-ACCTGA CCTGCCGTCTAGAA-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. PCR products were electrophoresed in a 1% neutral agarose gels and visualized by ethidium bromide staining.

Northern blot analysis. Total RNA (10 μg) was denatured, fractionated on formaldehyde-containing 1% agarose gels, transferred onto nylon membrane filters, and immobilized by UV irradiation. Hybridization was carried out at 65 °C in a solution containing 1 M NaCl, 1% *N*-lauroyl sarcosine, 7.5% dextran sulfate, 100 μg of heat-denatured salmon sperm DNA/ml, and the indicated radio-labeled probe DNA. After hybridization, filters were washed twice with 2× SSC/0.1% *N*-lauroyl sarcosine at room temperature followed by two washes with 0.1× SSC/0.1% *N*-lauroyl sarcosine at 50 °C, and exposed to an X-ray film with an intensifying screen at −70 °C.

Western blot analysis. Cells were washed with ice-cold PBS and lysed in 25 mM Tris–Cl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF) [20], and the extracts were sonicated for 10 s and centrifuged at 15,000 rpm for 10 min to remove insoluble materials. The protein concentrations were determined by the Bradford protein assay (Bio-Rad Laboratories). Equal amounts of protein (50 μg) were then loaded onto an SDS–polyacrylamide gel and electro-transferred onto a nitrocellulose membrane filter. The membrane filter was blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T), probed with a monoclonal anti-p73 (Ab-4, NeoMarkers), a monoclonal anti-p53 (DO-1, Oncogene Research Products), a monoclonal anti-$p27^{KIP1}$ (clone 57, Transduction Laboratories), a monoclonal anti-N-CAM (NCAM-OB11, Sigma), a monoclonal anti-Skp2 (1G12E9, Zymed Laboratories), a polyclonal anti-E2F1 (C-20, Santa Cruz Biotechnology), a polyclonal anti-$p21^{WAF1}$ (H-164, Santa Cruz Biotechnology), a polyclonal anti-$p57^{KIP2}$ (C-20, Santa Cruz Biotechnology), a polyclonal anti-Cdk2 (M2, Santa Cruz Biotechnology), a polyclonal anti-phospho Smad1/5 (a kind gift from Dr. A. Nakao), or a polyclonal anti-actin (20–33, Sigma) antibody, and then incubated with the horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories). Bound antibodies were detected with an ECL Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Immunoprecipitation. Whole cell lysates (300 μg of protein) were subjected to immunoprecipitation with the antibody against p73 (Ab-4, NeoMarkers). Protein G–Sepharose beads were added to collect immune complexes and the beads were washed five times in the lysis buffer. Immunoprecipitates were then analyzed by immunoblotting with the anti-p73 antibody (Ab-4, NeoMarkers).

In vitro kinase assay. Whole cell lysates (600 μg of protein) were immunoprecipitated with the antibody against Cdk2 (M2, Santa Cruz Biotechnology). The immunoprecipitates were recovered by brief centrifugation, washed with lysis buffer, and resuspended in kinase buffer (50 mM Tris–Cl, pH 7.5, 10 mM $MgCl_2$, and 1 mM dithiothreitol) containing 100 μg/ml of Histone H1 (Boehringer–Mannheim). Reaction mixtures were incubated at 30 °C for 30 min in the presence of [γ-^{32}P]ATP. Reactions were terminated with the SDS loading buffer and analyzed by 10% SDS–PAGE.

Results and discussion

Phosphorylation of Smad1/5 upon BMP2 treatment in human neuroblastoma-derived cell lines

To examine whether human neuroblastoma-derived cell lines could respond to BMP treatment, the

expression levels of BMP receptors (*BMPR-IA*, *BMPR-IB*, and *BMPR-II*) in SH-SY5Y and RTBM1 cells were analyzed by RT-PCR method. As shown in Fig. 1A, mRNAs for BMP type I and type II receptors were detected in both cell lines. It has been well documented that, upon BMP binding, catalytically activated forms of type I receptors can recognize and phosphorylate receptor-activated Smads (R-Smads) including Smad1, 5, and 8 [21,22]. We then analyzed whether Smad1/5 could be phosphorylated in the presence of BMP2. SH-SY5Y or RTBM1 cells were exposed to the recombinant human BMP2 (at a final concentration of 1 nM) and whole cell lysates prepared at the indicated time points after the BMP2 treatment were analyzed for phosphorylated Smad1/5 [23]. As shown in Fig. 1B, phosphorylated form of Smad1/5 was undetectable in untreated SH-SY5Y cells, but showed a remarkable increase at 30 min after the addition of BMP2, and a considerably high level was maintained even 2 h after the addition of BMP2. There were no significant changes in total amounts of Smad1/5 upon BMP2 treatment (data not shown). Similar results were also obtained in RTBM1 cells. These observations strongly suggest that there exist the intracellular machineries required to respond to BMP in human neuroblastoma-derived cell lines.

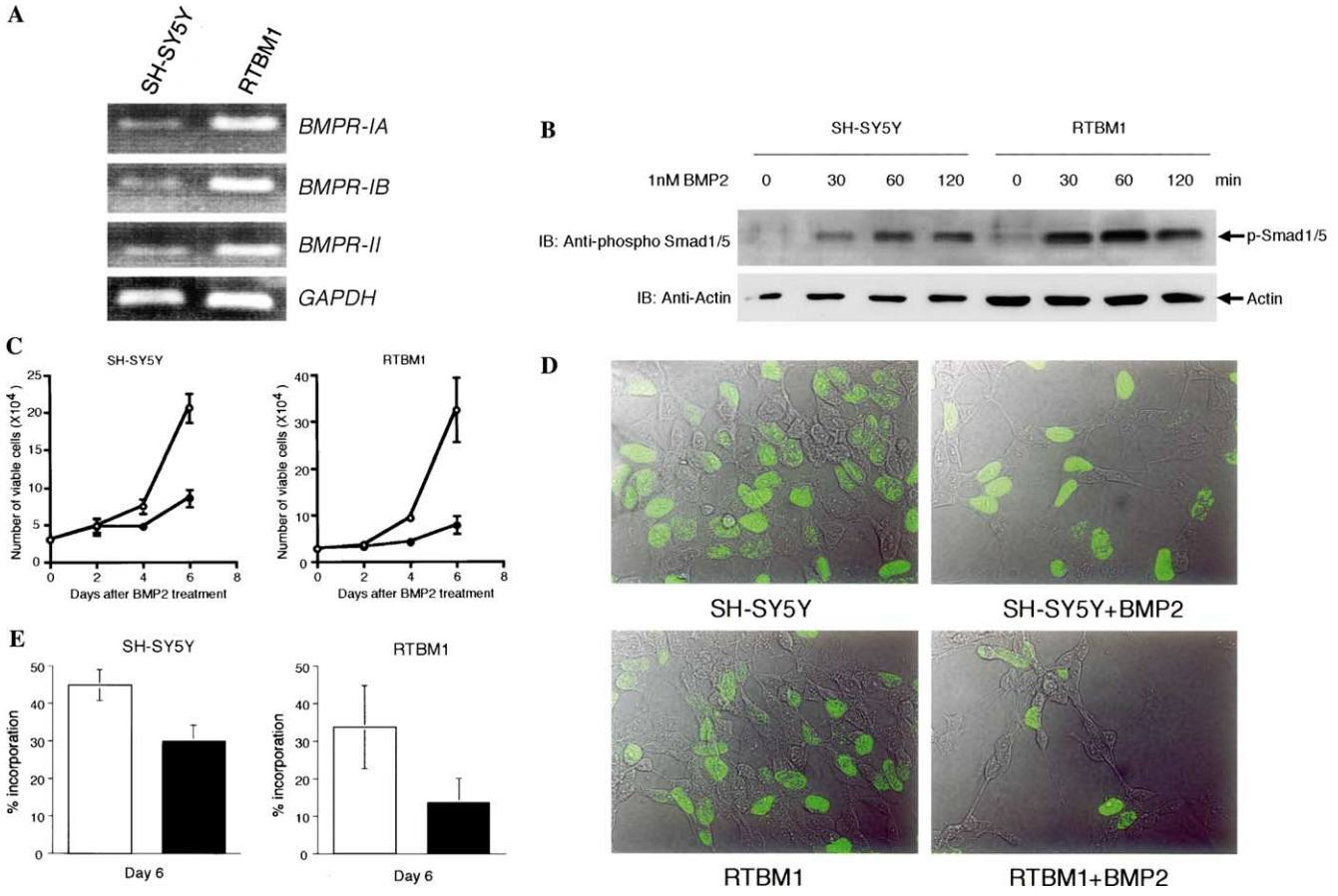

Fig. 1. Induction of growth inhibition by BMP2 in human neuroblastoma-derived cell lines. (A) Expression of BMP receptors (*BMPR*s) in human neuroblastoma SH-SY5Y and RTBM1 cell lines. Total RNA was extracted from each cell line and expression of *BMPR-IA*, *BMPR-IB*, and *BMPR-II* was analyzed by RT-PCR. Expression of *GAPDH* was used as an internal control. (B) Phosphorylation of Smad1/5. SH-SY5Y or RTBM1 cells were left untreated or treated with BMP2 (at a final concentration of 1 nM). Whole cell lysates prepared from SH-SY5Y (20 μg of protein) or RTBM1 (40 μg of protein) cells at the indicated time periods after the addition of BMP2 were subjected to immunoblotting with the antibody against phosphorylated forms of Smad1/5 (top panel). Actin protein was used as a control for loading and integrity (bottom panel). (C) Cell growth inhibition by BMP2 as determined by cell counts. SH-SY5Y (left panel) or RTBM1 (right panel) cells were grown in the absence (○) or presence (●) of BMP2 and the number of viable cells was counted in triplicate at the indicated time points. The data represent means ± SD from three independent experiments. (D) Suppression of BrdU incorporation by BMP2. SH-SY5Y (upper panels) or RTBM1 (lower panels) cells were grown in the absence (left panels) or presence (right panels) of BMP2. Six days after the treatment, cells were exposed to bromodeoxyuridine (BrdU; at a concentration of 10 μM) for 1 h and then stained with a monoclonal anti-BrdU antibody, followed by an FITC-conjugated goat anti-mouse IgG secondary antibody. The numbers of BrdU-positive cells were counted and expressed as a percentage of the total number of cells examined (E). A minimum of 300 cells/dish was examined. Data are means ± SD from three independent experiments.

Growth arrest and neuroblastoma cell differentiation upon BMP2 treatment

We next examined a possible effect(s) of BMP on neuroblastoma cell growth. SH-SY5Y or RTBM1 cells were incubated for 2, 4, and 6 days in the presence or absence of BMP2 and their growth rate was determined by counting the number of viable cells. As shown in Fig. 1C, SH-SY5Y cells treated with BMP2 grew at a much slower rate than untreated cells, similar to the patterns observed in RTBM1 cells. BMP2-treated cells were also analyzed for their ability to incorporate bromodeoxyuridine (BrdU). As shown in Figs. 1D and E, a significant decrease in the number of BrdU-positive cells was observed in BMP2-treated SH-SY5Y or RTBM1 cells compared with cells left untreated, suggesting that treatment with BMP2 causes a decreased S phase entry to arrest cells at G1 phase.

To monitor morphological changes induced by BMP2, BMP2-treated SH-SY5Y and RTBM1 cells were checked by phase-contrast microscopy. As shown in Figs. 2A and B, a weak outgrowth of neurites which made cell-to-cell connections was first detectable in SH-SY5Y cells after 4 days of BMP2 treatment, whereas an extensive neurite outgrowth was observed in RTBM1 cells stimulated with BMP2 for 2 days. The expression of neural cell adhesion molecule (N-CAM), which is a recognized marker of neuronal differentiation [13,24], was increased in response to BMP2 (Fig. 2C). Intriguingly, RTBM1 cells grew as aggregates and the formation of neurite outgrowth was much more prominent than that observed in SH-SY5Y cells following treatment with BMP2, suggesting that BMP2 has a lesser effect on morphological differentiation of SH-SY5Y cells than that of RTBM1 cells. As reported by Kawamura et al. [25] BMP2 caused the cell cycle arrest at G1 phase and subsequently induced apoptosis in human myeloma cells, however, we did not detect the apoptotic cell death in RTBM1 cells exposed to BMP2 (data not shown). In addition, the BMP2-dependent neurite outgrowth was undetectable in a neuroblastoma cell line, SK-N-AS, which expresses a barely detectable level of *BMPR*s (data not shown). Our results suggest that BMP2 induces neuronal differentiation in human neuroblastoma cell lines.

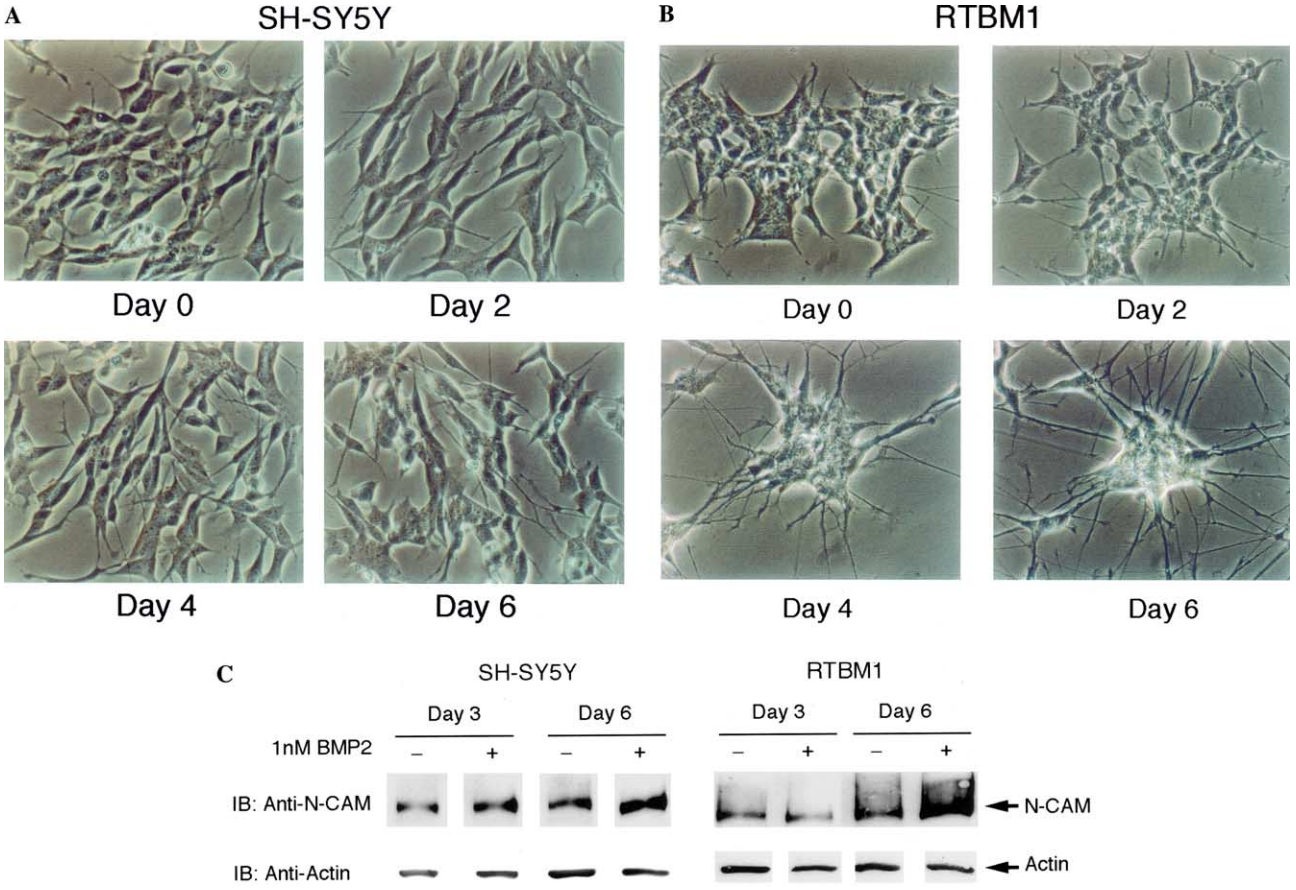

Fig. 2. Induction of neuronal differentiation by BMP2 in human neuroblastoma-derived cell lines. SH-SY5Y (A) or RTBM1 (B) cells were cultured in normal growth medium containing BMP2 (at a final concentration of 1 nM) and photographed at the indicated time periods after the addition of BMP2. (C) Induction of endogenous N-CAM by BMP2. At the indicated time points after the treatment with BMP2, whole cell lysates were prepared from SH-SY5Y (left panel) or RTBM1 (right panel) cells, and 20 μg of total protein was analyzed by immunoblotting with the anti-N-CAM antibody (top panels). Actin expression was examined as a loading control (bottom panels).

Expression of DAN and p53 family members is down-regulated during BMP2-induced neuroblastoma cell differentiation

As described previously, the expression of *DAN* was significantly increased during the retinoic acid (RA)-induced neuroblastoma cell differentiation, and overexpression of DAN resulted in an enhancement of this differentiation process [26]. Recently, it has been shown that p73, a newly identified p53 family member, plays a crucial role in the RA-dependent neuroblastoma differentiation [13]. We have recently shown that p73 transactivates the transcription of *DAN* [27]. To investigate whether functional interaction between DAN and p53 family members could be involved also in the regulation of the BMP-induced differentiation of neuroblastoma cells, we examined the expression levels of DAN and p53 family members including p73 and p53 in response to BMP2. Total RNA prepared from RTBM1 cells left untreated or treated with BMP2 for 1, 3, or 6 days was subjected to Northern blot analysis using a radio-labeled *DAN* cDNA as a probe. In contrast to RA-induced neuroblastoma differentiation, BMP2 treatment resulted in a significant down-regulation of *DAN* expression in a time-dependent manner (Fig. 3A). Considering that DAN can possibly attenuate BMP signaling [17,18], it seems likely that this down-regulation of *DAN* expression in response to BMP2 contributes in part to the transduction of its signal. Similarly, a remarkable reduction of p73α as well as p53 at a protein level was observed during BMP2-induced differentiation of RTBM1 cells (Fig. 3B). As described previously, RA treatment resulted in the down-regulation of *MYCN* expression in RTBM1 cells [26], whereas the expression level of *MYCN* remained constant in BMP2-treated RTBM1 cells (data not shown). These results suggest that both BMP2 and RA alone have an ability to induce neuronal differentiation in neuroblastoma-derived cell lines, however, the molecular mechanisms underlying the induction of neuroblastoma differentiation might differ between these two agents.

Accumulation of $p27^{KIP1}$ in response to BMP2

To elucidate the molecular mechanisms by which BMP induces the growth arrest and the neuronal differentiation in human neuroblastoma cells, we examined the effect of BMP2 on the expression levels of Cdk inhibitors and Cdk2 as well as E2F1. Whole cell lysates prepared from RTBM1 cells exposed to BMP2 for 2, 4, or 6 days were analyzed for the expressions of $p21^{WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$, $p16^{INK4a}$, Cdk2, and E2F1. As shown in Fig. 4A, BMP2 treatment induced a remarkable accumulation of $p27^{KIP1}$ in a time-dependent manner, whereas the amounts of $p57^{KIP2}$ and Cdk2 remained constant, regardless of BMP2 treatment. The accumulation of $p27^{KIP1}$ began to be observed as early as 2 days after BMP2 treatment, when the decrease of growth rate initiated. We then determined the effect of BMP2 on Cdk2 activity. Whole cell lysates derived from RTBM1 cells left untreated or exposed to BMP2 for 2, 4, or 6 days were immunoprecipitated with the anti-Cdk2 antibody, and the kinase activities of Cdk2 in each precipitate were analyzed by using Histone H1 as a substrate. As shown in Fig. 4B, a significant reduction of the Cdk2 activity was detected in the presence of BMP2. In our experimental conditions, $p16^{INK4a}$ was undetectable (data not shown). In contrast to $p27^{KIP1}$, the expression level of $p21^{WAF1}$ was significantly decreased in response to BMP2. This down-regulation of $p21^{WAF1}$, which is one of the direct targets regulated by p53 family members [28], might be due to the BMP2-dependent reduction of p73 and p53. Additionally, the amount of E2F1, which is required for the entry into S phase [29], was decreased in the presence of BMP2. These results suggest that $p27^{KIP1}$ plays a crucial role in the BMP2-induced growth arrest and neuronal differentiation of neuroblastoma cells.

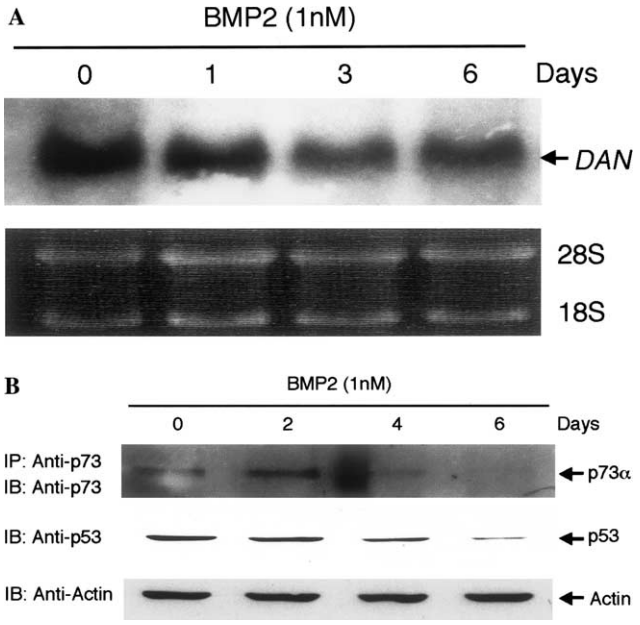

Fig. 3. Expression levels of *DAN* and p53 family members are decreased during neuroblastoma cell differentiation induced by BMP2. (A) Northern blot analysis of *DAN* expression. Total RNA was prepared from RTBM1 cells at the different times after the addition of BMP2 and subjected to Northern blotting with the radio-labeled human *DAN* cDNA. To control for RNA quality and integrity, the ethidium-bromide stained gel is shown in the lower panel. (B) Expression of p73α and p53 was analyzed by immunoblotting. After BMP2 treatment for the indicated times, whole cell lysates were prepared from RTBM1 cells and 50 μg of total protein was subjected to immunoblotting with the anti-p53 (middle panel) antibody. For p73α, whole cell lysates (300 μg of protein) from RTBM1 cells, untreated or treated with BMP2, were immunoprecipitated with the anti-p73 antibody, followed by immunoblotting with the anti-p73 antibody (top panel). Actin immunoblot was used as a loading control (bottom panel).

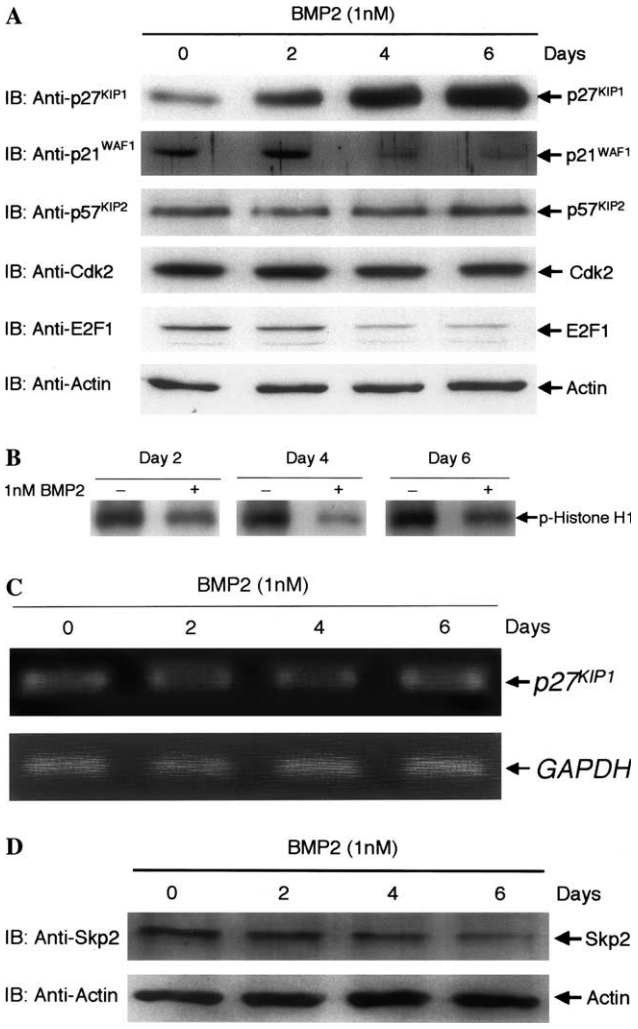

Fig. 4. $p27^{KIP1}$ accumulation during neuroblastoma cell differentiation induced by BMP2. (A) Induction of $p27^{KIP1}$ expression by BMP2. RTBM1 cells were treated with BMP2 for the indicated times and whole cell lysates (50 μg of protein) were analyzed by immunoblotting with the antibody specific for $p27^{KIP1}$ (first panel), $p21^{WAF1}$ (second panel), $p57^{KIP2}$ (third panel), Cdk2 (fourth panel), or E2F1 (fifth panel). Actin expression was examined as a loading control (sixth panel). (B) Interaction of $p27^{KIP1}$ with Cdk2. Whole cell lysates (600 μg of protein) prepared from RTBM1 cells left untreated or treated with BMP2 for the indicated time periods were immunoprecipitated with the anti-Cdk2 antibody and the immunoprecipitates were subjected to in vitro kinase assays. The phosphorylated Histone H1 is indicated by an arrow. (C) RT-PCR analysis of *$p27^{KIP1}$* expression. Total RNA was prepared from RTBM1 cells at the indicated time points after the addition of BMP2, reverse-transcribed into cDNA, and PCR-amplified using specific primers for *$p27^{KIP1}$* or *GAPDH*. Expression of *GAPDH* serves as an internal control. (D) Down-regulation of Skp2 expression in response to BMP2. Whole cell lysates were prepared from untreated RTBM1 cells or RTBM1 cells exposed to BMP2 for the indicated time periods, and 50 μg of total protein was analyzed by immunoblotting with the anti-Skp2 (top panel) or anti-actin (bottom panel) antibody.

Next, we examined whether the BMP2-dependent accumulation of $p27^{KIP1}$ could be regulated at transcriptional level. With the use of RT-PCR analysis, we measured the amount of *$p27^{KIP1}$* mRNA in RTBM1 cells exposed to BMP2. As shown in Fig. 4C, we observed negligible changes in *$p27^{KIP1}$* mRNA level regardless of BMP2 treatment, suggesting that BMP2 treatment might reduce the rate of $p27^{KIP1}$ degradation. Accumulating evidence suggests that S-phase kinase-associated protein 2 (Skp2) is required for the ubiquitin-mediated degradation of $p27^{KIP1}$ [30–32]. Recently, it has been shown that Skp2 expression was inversely correlated with $p27^{KIP1}$ expression in gastric carcinoma [33]. We therefore examined Skp2 expression in BMP2-treated RTBM1 cells. As shown in Fig. 4D, Skp2 was detected in proliferating RTBM1 cells, whereas the amount of Skp2 was reduced in response to BMP2 in a time-dependent manner, raising a possibility that BMP2-mediated signaling might contribute to cell fate determination of neuroblastoma cells by regulating the balance between intracellular levels of Skp2 and $p27^{KIP1}$.

It has been shown that BMP2 possesses an ability to induce neurogenesis in the neural crest cells from which neuroblastoma originates [34]. During the preparation of this article, Gomez-Santos et al. [35] reported that BMP2 or TGF-β treatment induced neuroblastoma cell differentiation in SH-SY5Y cells. In accordance with their findings, our present results suggest that human neuroblastoma-derived cell lines retain the BMP2-mediated signaling pathway responsible for growth arrest and neuronal differentiation. Thus, our studies, together with their findings, should provide an attractive therapeutic strategy in the treatment of neuroblastoma. In addition, our results demonstrate a first evidence showing that the selective induction of a key Cdk inhibitor $p27^{KIP1}$ is associated with this differentiation process. Accumulation of $p27^{KIP1}$ in response to BMP2 reduces the activity of Cdk2 and thereby inhibits the proliferative ability of cells, however, it remains unclear whether $p27^{KIP1}$ is directly involved in a pathway regulating the BMP2-induced neuroblastoma differentiation. Recently, it has been shown that the accumulation of $p27^{KIP1}$ is associated with RA-dependent neuroblastoma cell differentiation [11,12]. According to their results, RA treatment reduced the rate of ubiquitin/proteasome-mediated degradation of $p27^{KIP1}$. In RTBM1 cells exposed to BMP2, the amount of Skp2, which can promote the ubiquitin-mediated proteolysis of $p27^{KIP1}$ [30–32], was decreased at protein level in a time-dependent manner. It is very likely that this down-regulation of Skp2 enhances the stability of $p27^{KIP1}$, however, the precise molecular mechanism of the BMP2-induced reduction of Skp2 has yet to be established.

On the other hand, RA treatment of neuroblastoma cells induced an increase in the expression of DAN as well as p73 and a decrease in the expression of *MYCN* [13,26]. This is in marked contrast to the effect of BMP2 on the expression of those genes. Intriguingly, it has been reported that neuroblastoma cell differentiation was inhibited, when cells were treated with both BMP2

and RA, suggesting that these factors are antagonistic [35]. We have demonstrated that the expression level of *DAN* was increased in response to RA and overexpression of DAN enhanced the RA-mediated neurite outgrowth in SH-SY5Y cells [26]. Furthermore, Pearce et al. [18] reported that DAN acted as an inhibitor of BMP signaling in mammalian cells. Based on these observations, it is possible that there exist alternative signaling pathways required for RA- or BMP-mediated neuronal differentiation, which could be characterized by alterations of *DAN* expression.

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