

# Cyclic Adenosine Monophosphate (cAMP)-Induced Histone Hyperacetylation Contributes to its Antiproliferative and Differentiation-Inducing Activities

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Histone acetylation is linked to the control of chromatin remodeling, which is involved in cell growth, proliferation, and differentiation. It is not fully understood whether cyclic adenosine monophosphate (cAMP), a representative differentiation-inducing molecule, is able to modulate histone acetylation as part of its anticancer activity. In the present study, we aimed to address this issue using cell-permeable cAMP, *i.e.* dibutyl cAMP (dbcAMP) and C6 glioma cells. As reported previously, under the conditions of our studies, treatment with dbcAMP clearly arrested C6 cell proliferation and altered their morphology. Its antiproliferative and differentiation-inducing activity in C6 glioma cells involved upregulation of p21<sup>WAF/CIP1</sup>, p27<sup>kip1</sup>, glial fibrillary acidic protein (GFAP), and Cx43, as well as downregulation of vimentin. Furthermore, dbcAMP modulated the phosphorylation of ERK and Akt in a time-dependent manner and altered the colocalization pattern of phospho-Src and the actin cytoskeleton. Interestingly, dbcAMP upregulated the enzyme activity of histone acetyltransferase (HAT) and, in parallel, enhanced cellular acetyllysine levels. Finally, the hyperacetylation-inducing compound, sodium butyrate (NaB), a histone deacetylase (HDAC) inhibitor, displayed similar anticancer activity to dbcAMP. Therefore, our data suggest that antiproliferative and differentiation-inducing activities of dbcAMP may be generated by its enhanced hyperacetylation function.

**Key words:** Cyclic AMP, Antiproliferative Effect, Histone Acetylation

## Introduction

Since the discovery of histone acetylation by Allfrey and his colleagues (1964) more than four decades ago, a general correlation between core histone acetylation and transcription activity has now been well established: hyperacetylation of histones correlates with gene activation, while hypoacetylation correlates with gene repression (Countryman *et al.*, 2008). It was suggested that acetylation of lysine residues causes weaker interactions between histones and DNA, thereby facilitating the access of various transcriptional machinery molecules to the promoter region of DNA (Hong *et al.*, 1993). Several transcriptional coactivators with histone acetyltransferase (HAT) activity have been suggested, and these

coactivators cooperate with histone deacetylase (HDAC) to regulate gene expression. Recently, several HDAC inhibitors such as sodium butyrate (NaB), pyroxamide, and trichostatin have been identified and were found to modulate the chromatin structure and induce the differentiation of many tumour cell lines, including C6 cells, and their growth arrest by hyperacetylation (Butler *et al.*, 2001; Kamitani *et al.*, 2002). Due to these biochemical features, HDAC inhibitors have been suggested as next-generation anticancer drugs. Although the functional mechanisms of the role of histone acetylation in the anticancer effect have not yet been clearly elucidated, histone acetylation has been extensively investigated in the field of cancer research.

C6 glioma cells, derived from rat brains treated with *N*-nitro-methylurea (Benda *et al.*, 1968), may differentiate into either oligodendrocytes or

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astrocytic cells or astrocytes (Kumar *et al.*, 1986; Mangoura *et al.*, 1989; Parker *et al.*, 1980). Astrocytes are the most abundant glia type in the central nervous system, and they fulfill various important functions, including support and protection of neurons, induction of neurogenesis, regulation of synapse formation and transmission, and initiation of immune responses (Aschner, 1998; Mucke and Eddleston, 1993). Agents that increase the intracellular levels of cyclic adenosine monophosphate (cAMP), such as *N*<sup>6</sup>,2'-*O*-dibutyryl-adenosine 3',5'-cyclic AMP (dbcAMP), may induce the differentiation of C6 cells into astrocytes via activation of p38 mitogen-activated protein kinase (Thomas *et al.*, 2000).

Differentiation of astrocytes is accompanied by morphological changes concomitant with redistribution of the cytoskeleton and an increase of glial fibrillary acidic protein (GFAP) synthesis. The latter intermediate filament protein is the major cytoskeletal component of astrocytes, and its synthesis is observed during maturation of glioblasts, during differentiation induced by neuronal interaction, and under pathological conditions (Dahl, 1981). The molecular mechanisms of astrocyte differentiation are poorly defined; nevertheless, they involve a shift in the synthesis of vimentin toward GFAP (Dahl *et al.*, 1986), which is required for astrocytic cells to acquire the structural phenotype (Chen *et al.*, 1994; Weinstein *et al.*, 1991). In particular, the contribution of cAMP to the modification of this change has not yet been understood in terms of the hyperacetylation of histones. In the present study, we investigated the roles of histone acetylation in cAMP-induced differentiation and growth arrest.

## Material and Methods

### Materials

[<sup>3</sup>H]-Acetic acid (7 Ci/mmol), [<sup>3</sup>H]-labeled thymidine, and *S*-adenosyl-L-[methyl-<sup>3</sup>H] methionine (85 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Rat C6 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and all cell culture reagents including fetal bovine serum (FBS) were purchased from GIBCO BRL (Gaithersburg, MD, USA). Antibodies to total and phospho-forms of acetylated histone H3, histone H3, Cx43, p21, p27, p53, Src, p85, Akt, ERK,

p38, JNK, and I $\kappa$ B $\alpha$  were purchased from Cell Signaling (Beverly, MA, USA), Upstate Biotechnology (Waltham, MA, USA), and Sigma-Aldrich, DAKO (Glostrup, Denmark), respectively.

### Cell culture

Rat glioma C6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were collected and passaged using a 0.05% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) solution, and cells were counted in triplicate with a hemocytometer.

### Cell proliferation assay

The MTT assay used to determine cell growth is based on the mitochondrial conversion of the water-soluble yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a water-insoluble purple formazan (Jun *et al.*, 2010). C6 cells were plated at a density of  $5 \cdot 10^4$  cells/well into 96-well tissue culture plates and pre-incubated overnight at 37 °C. After dbcAMP treatment, cells were incubated with MTT (0.5  $\mu$ g/ml) for 4 h at 37 °C. The formazan product was solubilized in 100  $\mu$ l of dimethyl sulfoxide (DMSO) and 20  $\mu$ l of Sorenson's solution (0.1 M glycine, 0.1 M NaCl, pH 10.2). The absorbance was measured at 490 nm with a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA).

### [<sup>3</sup>H]-Thymidine incorporation assay

Cell proliferation was also measured by [<sup>3</sup>H]-labeled-thymidine incorporation. In a 12-well tissue culture plate,  $1 \cdot 10^5$  cells were plated and treated each with NaB and dbcAMP as indicated. Subsequently, the cells were pulse-labeled with [<sup>3</sup>H]-labeled thymidine (2  $\mu$ Ci/well) for a final 6 h. The cells were washed twice with phosphate-buffered saline (PBS), twice with 5% (w/v) trichloroacetic acid (TCA), and once with 95% (v/v) ethanol. TCA-insoluble material was dissolved overnight in 0.2 M sodium hydroxide, and then the radioactivity was measured with a scintillation counter.

### Induction of C6 cell differentiation

In order to induce differentiation of C6 cells (Ko *et al.*, 2010), these were treated with 1 mM dbcAMP in serum-free DMEM for 72 h.

### *Assay of histone acetyltransferase activity*

To assay the histone acetyltransferase activity, P81 filter retention assays were carried out as previously described (Yang *et al.*, 2003). Briefly, a 6 cm × 8 cm P81 filter paper (Whatman, Maidstone, Kent, UK) was cut out and placed on two 6 cm × 8 cm Whatman 3MM filters. With a pencil and a clean ruler, 2 cm × 2 cm squares were drawn on the P81 filter and labeled with numbers. Acetylation reactions were set up in 0.5-ml microcentrifuge tubes on ice. Each contained 1  $\mu$ l of histone substrate, 4  $\mu$ l of sample buffer [250 mM Tris-HCl (pH 8.0), 50% glycerol, 5 mM *dl*-dithiothreitol (DTT), 0.5 mM EDTA, 5 mM phenylmethanesulfonyl fluoride (PMSF), 50 mM NaB], 74 nCi of [<sup>3</sup>H]-acetyl-CoA (10 Ci/mmol), and nuclear lysates. The tubes were gently tapped, briefly centrifuged, and transferred to a 37-°C water bath. After 10 min, the tubes were put back on ice. The total reaction mixtures were immediately spotted onto the P81 filter paper prepared above; one 2 cm × 2 cm square was used for spotting one reaction mixture. The filter paper was air-dried for 30 min and washed in 50 mM sodium carbonate (pH 9.2) with agitation in a reciprocal shaking water bath (70 rpm; Precision, Chicago, IL, USA) at 37 °C. After drying for 1 h, each filter paper was cut out, and radioactivity was counted in 5 ml of liquid scintillation fluid in a liquid scintillation counter (LS 6500; Beckman, Palo Alto, CA, USA).

### *Assay of histone deacetylase activity*

In order to assay the HDAC activity, radiolabeled acetylated histone substrates were used (Davie *et al.*, 2003). Briefly, the nuclear lysates were incubated in an 1.5-ml tube in a final volume of 0.3 ml, containing 25 mM sodium phosphate buffer, pH 7.0, and 100  $\mu$ g (approximately 10 cpm) of [<sup>3</sup>H]-acetate-labeled histones. The reaction mixtures were incubated for 1 h at 37 °C with shaking, and the reaction was then terminated by adding 30  $\mu$ l of 10 M HCl. The radioactive acetate released by the deacetylase was extracted by adding 0.6 ml ethyl acetate and mixing. After centrifugation at 16,000 × *g* for 1 min in a microcentrifuge, 0.3 ml of the upper phase was transferred into 5 ml of scintillation liquid for radioactivity counting.

### *Immunoblotting*

C6 cells were homogenized in lysis buffer, containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium pyrophosphate, 30 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM PMSF. After their protein concentration was determined, the lysates were boiled in Laemmli sample buffer for 3 min, and 30  $\mu$ g of protein were subjected to sodium dodecyl (SDS)-polyacrylamide gel electrophoresis (PAGE) on 15% slab gels as reported previously (Chung *et al.*, 2010a; Rhee *et al.*, 2010). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Life Sciences, Arlington Heights, IL, USA), and the membranes were blocked for 30 min in TBS containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder; then they were incubated overnight with primary antibodies raised against Cx43, p21, p27, p53, Src, p85, Akt, ERK, p38, JNK, and I $\kappa$ B $\alpha$ , respectively. The membranes were then washed with TBS-T and incubated for 1 h with an anti-rabbit (Amersham Life Sciences) or an anti-mouse (ZYMED Laboratories, San Francisco, CA, USA) secondary antibody. Bound antibodies were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences).

### *Reverse transcription polymerase chain reaction (RT-PCR)*

C6 cells were plated at a density of 1 × 10<sup>5</sup> cells/well in 6-well tissue culture plates. After treatment with the reagents indicated, the total RNAs were obtained using Trizol reagent (GIBCO BRL, Grand Island, NY, USA). After determining the amount of total RNA followed by electrophoresis on formaldehyde-agarose gel, cDNA synthesis was performed using MMLV RTase (SuperBio, Seoul, Korea) as reported previously (Chung *et al.*, 2010b; Yang *et al.*, 2010). The PCR was performed using Super Taq polymerase (SuperBio) in 20  $\mu$ l of reaction mixture containing 2  $\mu$ l of reaction buffer, 1  $\mu$ l of 5 mM dNTPs, 1  $\mu$ l each of forward and reverse primers, and 1  $\mu$ l of template cDNA. The sequences of the primers used were as follows; GFAP: forward, 5'-atgcaagaacagaagagtgtatc-3', and reverse, 5'-gcttaacgttgagtatccttgga-3'; vimentin: forward, 5'-gtcattcagacaggatgtgacaat-3', and reverse, 5'-atctcttctcatgttcttgatct-3'; glycer-

aldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-cctggccaaggtcatcat-3', and reverse, 5'-gccatgtaggcatgaggt-3'. The RT-PCR products were separated on a 1% agarose gel containing 0.5  $\mu$ l/ml ethidium bromide, and the images were captured using Bioimage Processing System (Bio-medlab, Seoul, Korea).

### Statistical analysis

Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means  $\pm$  standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. *p* values of 0.05 or less were considered to be statistically significant.

## Results and Discussion

cAMP is known as a differentiation-inducing agent that causes cell growth arrest, apoptosis, and reversion of the transformed phenotype of cancer cells (Cho-Chung, 1990). Indeed, it has been found that astrocyte differentiation is accompanied by elevation of intracellular cAMP (Chen *et al.*, 1996). The exact mechanism of these events remains to be elucidated. In this study, we explored the potential involvement of histone

acetylation in cAMP-induced glioma cell differentiation using the cell-permeable cAMP derivative dbcAMP, C6 glioma cells, and several biochemical experimental methods such as enzyme assays, confocal microscopy, and immunoblotting.

First, under our conditions, we confirmed whether dbcAMP was able to arrest the proliferation of C6 glioma cells by measuring their viability. dbcAMP (1 mM) treatment strongly suppressed the normal growth and proliferation of C6 cells according to both MTT reduction (Fig. 1A) and [ $^3$ H]-thymidine incorporation (Fig. 1B) assays. Initial cell numbers were maintained by dbcAMP treatment, suggesting that the growth arrest pattern is not simply due to its cytotoxicity. It has previously been reported that dbcAMP induces the differentiation of C6 glioma cells (Moreno *et al.*, 2006). Fig. 2 shows the morphological changes seen in differentiated astrocytes after dbcAMP exposure; the bordered and extruded long, branched cytoplasmic processes of the astrocytes retreated and elongated filamentous protrusions were formed (Fig. 2A). In addition, the altered levels of several differentiation marker proteins such as vimentin, GFAP, and Cx43 strongly indicated its differentiation-inducing activity. Specifically, dbcAMP strongly upregulated the protein and mRNA levels, respectively, of GFAP (Fig. 2B and 2C) and Cx43 (Fig. 2D), while that of vimentin was decreased (Fig. 2C). The treatment with

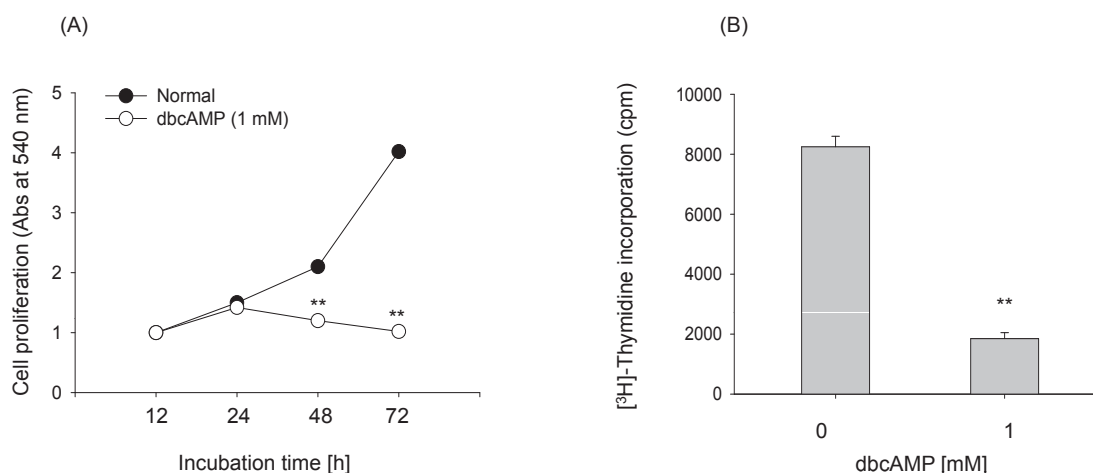


Fig. 1. Effect of dbcAMP on C6 cell proliferation. (A) C6 glioma cells ( $1 \cdot 10^5$  cells/ml) were incubated with dbcAMP for the indicated times. Cell growth was measured by the MTT assay, as described in Materials and Methods. (B) The effect of dbcAMP on the proliferation of C6 glioma cells ( $1 \cdot 10^5$  cells/ml) was examined by the [ $^3$ H]-thymidine incorporation assay. Data are means  $\pm$  SEM for triplicate experiments. \*\* *p* < 0.01 compared to normal group.



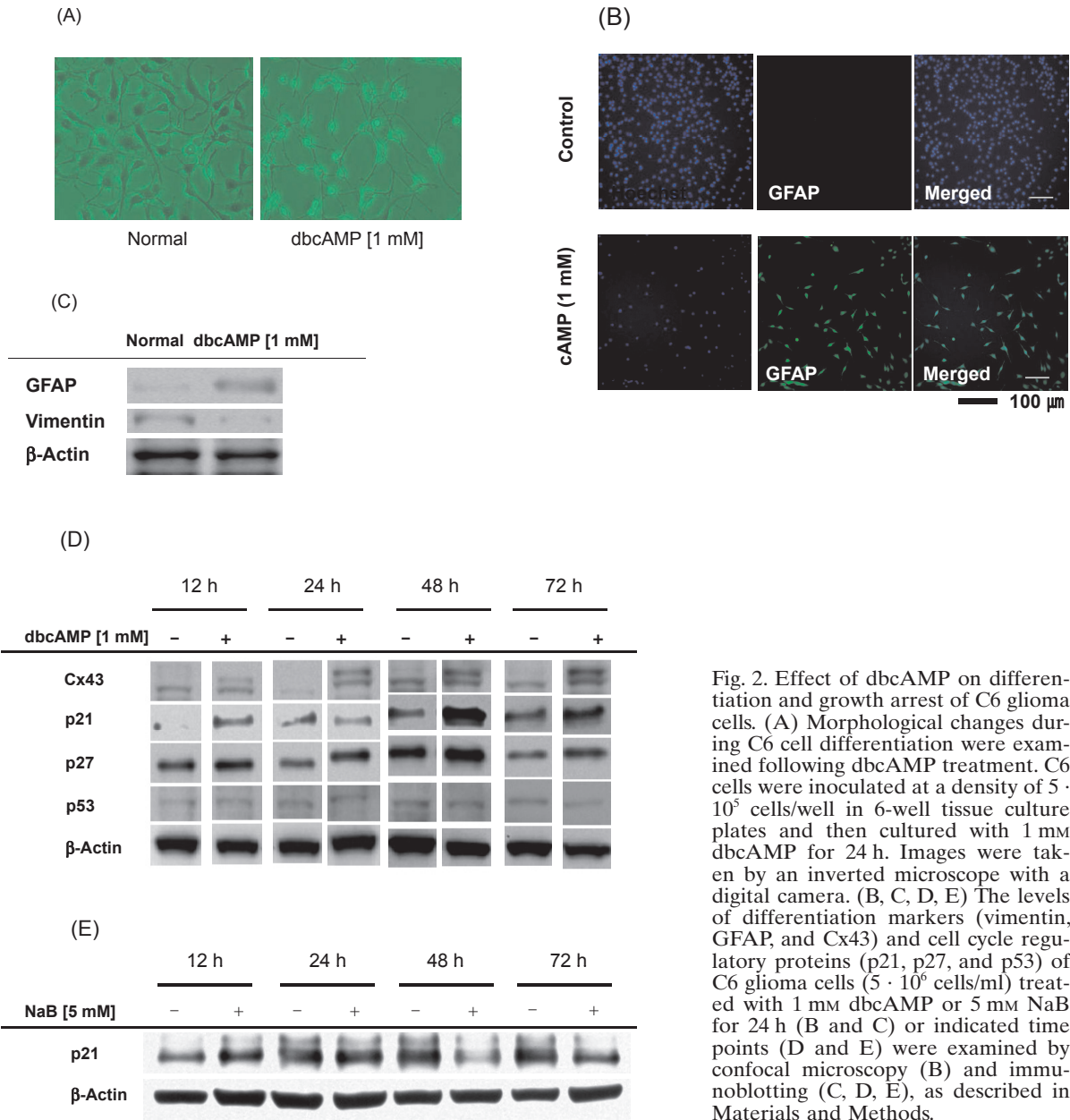


Fig. 2. Effect of dbcAMP on differentiation and growth arrest of C6 glioma cells. (A) Morphological changes during C6 cell differentiation were examined following dbcAMP treatment. C6 cells were inoculated at a density of  $5 \cdot 10^5$  cells/well in 6-well tissue culture plates and then cultured with 1 mM dbcAMP for 24 h. Images were taken by an inverted microscope with a digital camera. (B, C, D, E) The levels of differentiation markers (vimentin, GFAP, and Cx43) and cell cycle regulatory proteins (p21, p27, and p53) of C6 glioma cells ( $5 \cdot 10^6$  cells/ml) treated with 1 mM dbcAMP or 5 mM NaB for 24 h (B and C) or indicated time points (D and E) were examined by confocal microscopy (B) and immunoblotting (C, D, E), as described in Materials and Methods.

dbcAMP also strongly increased the protein level of p21<sup>WAF/CIP</sup> and slightly enhanced the level of p27<sup>kip1</sup> from 12 to 72 h, whereas it had no effects on the level of p53, a tumour suppressor gene (Fig. 2D). NaB, a histone deacetylase inhibitor and a well-known anticancer drug, also enhanced the p21 level at 12 and 24 h, although the p21 protein was decreased at 48 and 72 h (Fig. 2E).

Since differentiation and cell growth arrest are closely related to intracellular signaling cascades, including the cell survival signaling cascade composed of Src, PI3K, Akt, and MAPK (ERK, p38, and JNK) (Bhattacharjee *et al.*, 2004), the modulatory role of dbcAMP was carefully examined. Exposure to dbcAMP for up to 24 h remarkably reduced the phosphorylation of Src, Akt, and ERK

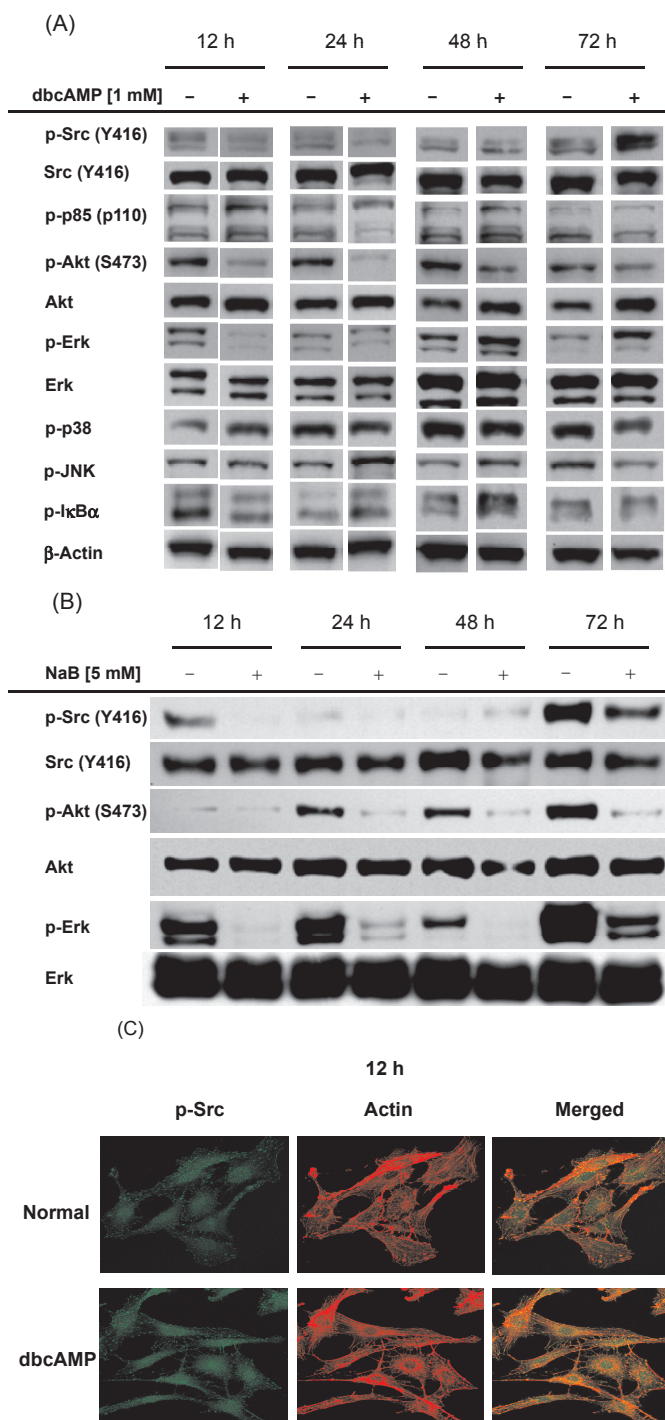


Fig. 3. Effect of dbcAMP on cell survival signaling in C6 glioma cells. (A, B) The levels of cell survival signaling enzymes (Src, p85/PI3K, Akt, IκBα, ERK, p38, and JNK) of C6 glioma cells ( $5 \cdot 10^6$  cells/ml) treated with 1 mM dbcAMP or 5 mM NaB for the indicated times were examined by immunoblotting analysis as described in Materials and Methods. (C) The localization of phospho-Src and actin in C6 glioma cells was examined by confocal microscopy.

without altering the total levels of these proteins, whereas ERK phosphorylation was enhanced at 48 and 72 h (Fig. 3A). Similarly, NaB also suppressed the levels of phospho-Src (p-Src), phospho-Akt (p-Akt), and phospho-ERK (p-ERK) at 12, 24, and 48 h (Fig. 3B). Furthermore, upon induction of differentiation, cells undergo morphological changes from a bipolar to a satellite shape concomitantly with a shift in the expression of the intermediate filament protein vimentin towards GFAP (Roymans *et al.*, 2000). Because it has been demonstrated that there is tight regulation between Src and the actin cytoskeleton during this morphological change (Kim *et al.*, 2010; Stautz *et al.*, 2010; Winograd-Katz *et al.*, 2010), we also investigated the localization pattern between p-Src and the actin cytoskeleton by confocal microscopic analysis. Although the protein level of p-Src at 12 h was slightly reduced in the immunoblotting data, Fig. 3B clearly exhibits the enhanced level of p-Src in the nuclear area. In contrast, the actin cytoskeleton was localized at the perinuclear site, suggesting that the molecular interaction between actin and p-Src could be interrupted by dbcAMP (Fig. 3C). The functional significance of Src in proliferative and tumorigenic responses has been widely reported. Thus, siRNA-mediated inhibition of Src or application of PP2, a specific inhibitor of kinases of the Src family, have been shown to reduce the viability of MDA-MB-435S, ovarian carcinoma, and pancreatic ductal adenocarcinoma, respectively (Bjorge *et al.*, 2011; Nagaraj *et al.*, 2010) and to induce differentiation of neuroblastoma cells (Hishiki *et al.*, 2011; Tarui *et al.*, 2010). Therefore, it is considered that alteration or inhibition of the Src activity is involved in the regulation of proliferation and differentiation by dbcAMP in C6 glioma cells.

Control of core histone tail acetylation is considered one of many powerful strategies to block tumour cell growth and proliferation and to induce their differentiation (Balakin *et al.*, 2007). Although the molecular mechanism of the anticancer response to histone hyperacetylation is not fully understood, the upregulation of the transcriptional control in cell cycle regulatory proteins and differentiation-relevant proteins seems to be a major mechanism in this event. For these reasons, HDAC inhibitors such as NaB, pyroxamide, and trichostatin have been found to upregulate p21<sup>WAF/CIP</sup>, a cyclin-dependent kinase inhibitor that inhibits RB protein phosphorylation, which

is linked to inhibition of the G<sub>1</sub>-S phase transition (Liu and Yamauchi, 2009); in parallel, HDAC inhibitors have also been linked to the indirect inhibition of cyclin A and cyclin D functions (Johnstone and Licht, 2003; Rosato *et al.*, 2003). To determine the involvement of hyperacetylation in dbcAMP-induced C6 glioma cell differentiation, the effect of dbcAMP on histone acetylation was tested using purified histones. As can be seen in Fig. 4A, dbcAMP enhanced the activity of HAT by 160%, whereas NaB did not change it. In contrast, NaB strongly blocked the enzyme activity of HDAC up to 80%, whereas the HDAC activity was not affected by dbcAMP exposure (Fig. 4B). This effect was confirmed using an anti-acetylsine antibody. Thus, NaB and dbcAMP treatment enhanced the level of acetylsine in the nucleus compared to normal cells, based both on confocal microscopy (Fig. 4C) and immunoblotting analysis at 24 h (Fig. 4D). Histone methylation was also clearly diminished by these two inhibitors (Fig. 4E), suggesting that the hyperacetylation of histones induced by these compounds can affect another histone post-translational modification, as reported previously (Sarg *et al.*, 2004). The similarity of the features evoked by these compounds was also confirmed by measuring the effect of NaB on cell proliferation and the expression pattern of structural proteins as markers of astrocytic differentiation. NaB arrested the cell proliferation, maintaining initial cell numbers and blocking [<sup>3</sup>H]-thymidine incorporation (Figs. 5A and 5B). This compound also increased the transcript level of GFAP and conversely decreased the vimentin transcript level (Fig. 5C), implying that growth arrest and induction of differentiation of C6 glioma cells are due to hyperacetylation of histone proteins, as previously suggested (Engelhard *et al.*, 2001; Li *et al.*, 2005). Although the precise underlying mechanism of such anticancer activities of hyperacetylation in C6 glioma cells is poorly understood, it has been reported that HDAC inhibitors such as NaB, pyroxamide, and trichostatin could modulate the chromatin structure and induce the differentiation of many tumour cell lines, including C6 cells (Butler *et al.*, 2001; Engelhard *et al.*, 2001; Joung *et al.*, 2012; Kamitani *et al.*, 2002).

In conclusion, we have found that dbcAMP-mediated anticancer effects, such as arrest of the cell cycle, changes in the cell morphology, and induction of differentiation of C6 glioma cells, were

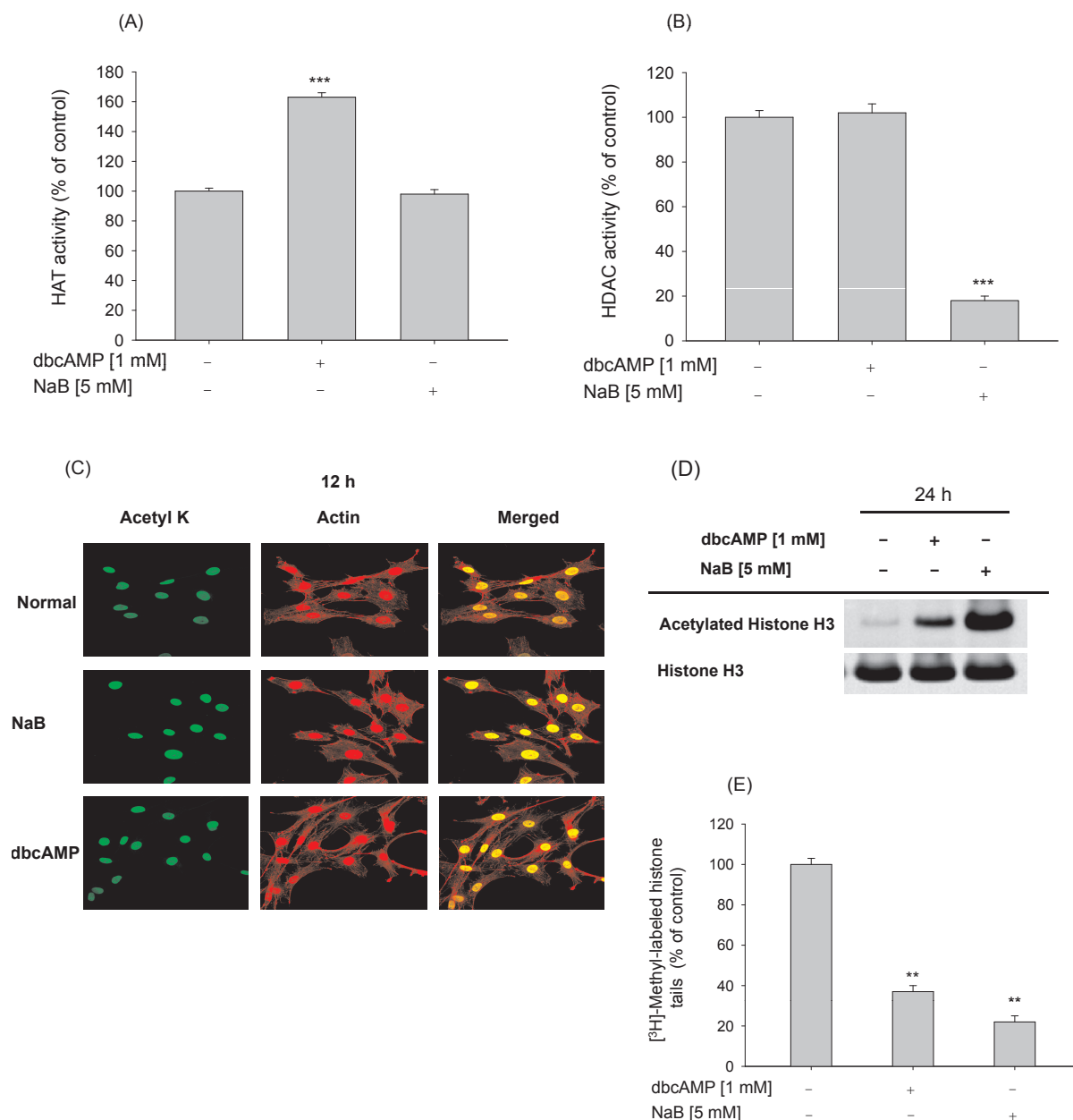


Fig. 4. Effect of dbcAMP on histones and cellular hyperacetylation and methylation. (A) Histone acetyltransferase (HAT) activity was measured using P81 filter paper spotted with acetylated core histones in nuclear extracts containing histone acetyltransferase and [<sup>3</sup>H]-acetyl-CoA as an acetyl donor. (B) Histone deacetylase (HDAC) activity was measured by detecting the radioactive acetate liberated from [<sup>3</sup>H]-acetyl-labeled histones by nuclear extracts containing histone deacetylase. After treatment of C6 cells with dbcAMP (1 mM) or NaB (5 mM) and [<sup>3</sup>H]-acetate for 24 h, histones were isolated and the [<sup>3</sup>H]-acetyl-labeled histones were determined. (C) The localization of acetyllysine (K) and actin in C6 glioma cells treated with dbcAMP (1 mM) was examined by confocal microscopy. (D) The levels of acetylated histone H3 and histone H3 from C6 glioma cells ( $5 \times 10^6$  cells/ml) treated with 1 mM dbcAMP or 5 mM NaB for 24 h were examined by immunoblotting analysis as described in Materials and Methods. (E) After treatment of C6 cells with NaB and [<sup>3</sup>H]-SAM for 24 h, histones were isolated, and [<sup>3</sup>H]-methyl radioactivity was counted. Data are means  $\pm$  SEM for triplicate experiments. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  compared to normal group.



related to upregulation of p21<sup>WAF/CIP</sup> and GFAP, downregulation of ERK and Akt, and alteration of p-Src and actin colocalization. Based on our observations that dbcAMP enhanced the HAT activity and acetyllysine level, and that NaB-mediated hyperacetylation resulted in similar anticancer activities, our data suggests that the anti-

cancer activity of dbcAMP is a consequence of its hyperacetylation-inducing activity.

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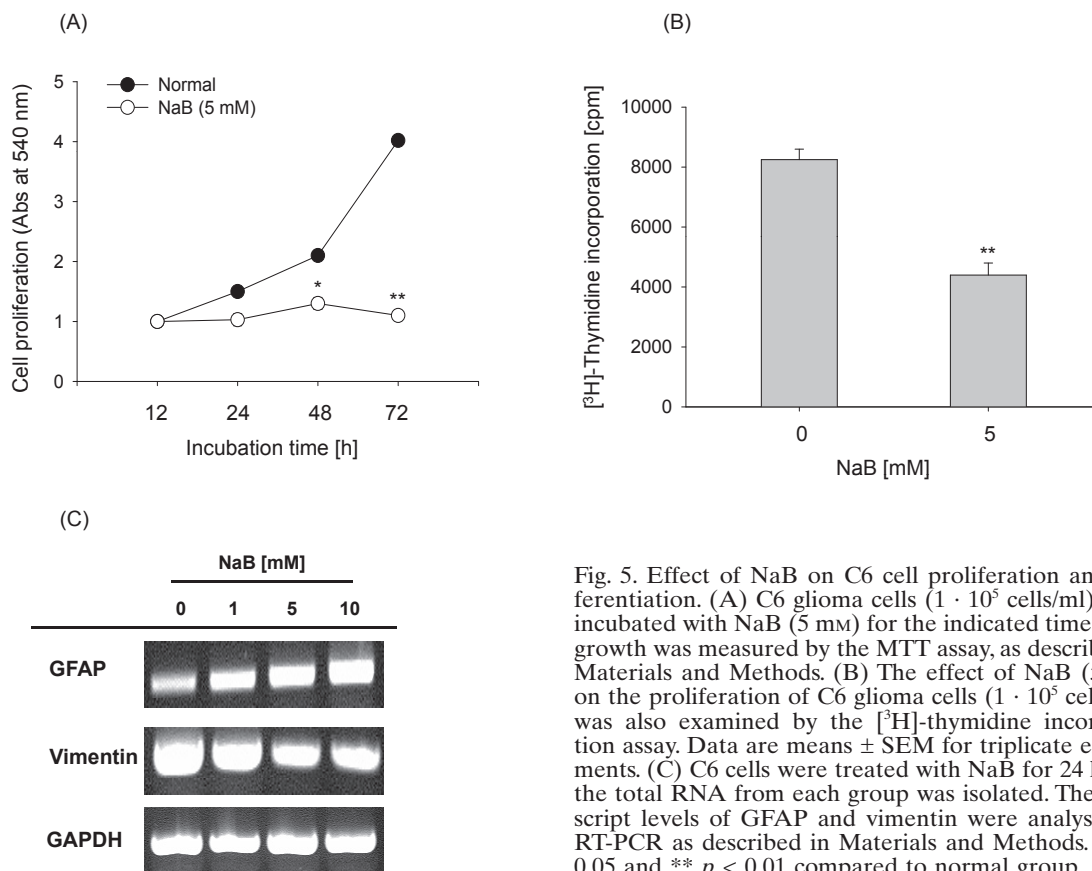


Fig. 5. Effect of NaB on C6 cell proliferation and differentiation. (A) C6 glioma cells ( $1 \cdot 10^5$  cells/ml) were incubated with NaB (5 mM) for the indicated times. Cell growth was measured by the MTT assay, as described in Materials and Methods. (B) The effect of NaB (5 mM) on the proliferation of C6 glioma cells ( $1 \cdot 10^5$  cells/ml) was also examined by the [ $^3$ H]-thymidine incorporation assay. Data are means  $\pm$  SEM for triplicate experiments. (C) C6 cells were treated with NaB for 24 h, and the total RNA from each group was isolated. The transcript levels of GFAP and vimentin were analysed by RT-PCR as described in Materials and Methods. \*  $p < 0.05$  and \*\*  $p < 0.01$  compared to normal group.

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