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Involvement of Akt2/protein kinase B β (PKBβ) in the 8-Cl-cAMP-Induced Cancer Cell Growth Inhibition

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8-chloro-cyclic AMP (8-Cl-cAMP), which induces differentiation, growth inhibition, and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. However, the exact mechanism of 8-Cl-cAMP functioning in cancer cells is not fully understood. Akt/protein kinase B (PKB) genes (Akt1, Akt2, and Akt3) encode enzymes belonging to the serine/threonine-specific protein kinase family. It has been suggested that Akt/PKB enhances cell survival by inhibiting apoptosis. Recently, we showed that 8-Cl-cAMP and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibited cancer cell growth through the activation of AMPK and p38 MAPK. Therefore, we anticipated that the phosphorylation of Akt/PKB would be decreased upon treatment with 8-Cl-cAMP. However, treatment with 8-Cl-cAMP and AICAR induced the phosphorylation of Akt/PKB, which was inhibited by ABT702 (an adenosine kinase inhibitor) and NBTI (an adenosine transporter inhibitor). Furthermore, whereas Compound C (an AMPK inhibitor), AMPK-DN (AMPK-dominant negative) mutant, and SB203580 (a p38 MAPK inhibitor) did not block the 8-Cl-cAMP-induced phosphorylation of Akt/PKB, TCN (an Akt1/2/3 specific inhibitor) and an Akt2/PKBβ-targeted siRNA inhibited the 8-Cl-cAMP- and AICAR-mediated phosphorylation of AMPK and p38 MAPK. TCN also reversed the growth inhibition mediated by 8-Cl-cAMP and AICAR. Moreover, an Akt1/PKBα-targeted siRNA did not reduce the phosphorylation of AMPK and p38 MAPK after treatment with 8-Cl-cAMP. These results suggest that Akt2/PKBβ activation promotes the phosphorylation of AMPK and p38 MAPK during the 8-Cl-cAMP- and AICAR-induced growth inhibition.

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8-chloro-cyclic AMP (8-Cl-cAMP) is one of the site-selective cAMP analogs that induce growth inhibition, apoptosis, reverse transformation, and differentiation in various cancer cell lines (Tagliaferri et al., 1988a, 1988b; Tortora et al., 1991; Kim et al., 2000). Although 8-Cl-cAMP inhibits cell proliferation and promotes apoptosis in cancer cells, it does not inhibit the growth of non-transformed cells (Ciardiello et al., 1990; Kim et al., 2001).

Conversion of 8-Cl-cAMP to 8-Cl-adenosine, one of the metabolites of 8-Cl-cAMP, is essential for its growth inhibitory effect (Van Lookeren Campagne et al., 1991; Langeveld et al., 1992; Lange-Carter et al., 1993; Gandhi et al., 2001; Ahn et al., 2004, 2005; Han et al., 2009). Furthermore, adenosine deaminase, which converts 8-Cl-adenosine to 8-Cl-inosine, was shown to block the cytotoxic effects of 8-Cl-cAMP, 8-Cl-AMP and 8-Cl-adenosine, and 5-(p-nitrobenzyl)-6-thio-inosine (NBTI), an inhibitor of adenosine uptake, and A134974, a selective adenosine kinase inhibitor were found to abrogate the 8-Cl-cAMP-induced inhibition of growth in cancer cells (Halgren et al., 1998; Ahn et al., 2004, 2005; Han et al., 2009). These studies demonstrate that the conversion of 8-Cl-cAMP to its metabolite, 8-Cl-adenosine, is required for its growth inhibitory effect in cancer cells.

We have previously reported that the conversion of 8-Cl-cAMP to 8-Cl-adenosine was indispensable for 8-Cl-cAMP-induced growth inhibition and apoptosis of cancer cells. In addition, the regulation of cancer cell growth mediated by 8-Cl-cAMP and 8-Cl-adenosine involved the activation of protein kinase C (PKC), Rap1 and p38 mitogen activated protein kinase (MAPK) in mouse fibroblast DT cells (Ahn et al., 2004, 2006). We further demonstrated that AMP-activated protein kinase (AMPK) activation acted upstream of p38 MAPK in HeLa cells during the course of 8-Cl-cAMP treatment (Ahn et al., 2005; Han et al., 2009).

Akt/protein kinase B (PKB) was identified in 1991 as a serine/threonine-specific protein kinase (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991b). The first characterized Akt/PKB was named Akt1/PKBα, and two very similar serine/threonine kinases, Akt2/PKBβ (Jones et al., 1991a) and Akt3/PKBγ (Konishi et al., 1995), were subsequently identified. Akt/PKB is activated by a wide variety of growth factors, including platelet-derived growth factor, epidermal growth factor, insulin, thrombin, and nerve growth factor (Downward, 1998). Phosphoinositide 3-kinase (PI3K) was known to regulate the activation of Akt/PKB upon

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stimulation with several growth factors (Burgering and Coffer, 1995; Franke et al., 1995) through the activation of two protein kinases, phosphoinositide-dependent kinase1 (PDK1) and phosphoinositide-dependent kinase2 (PDK2). The activated PDK1 and PDK2 add phosphates to Thr308 and Ser473 in Akt/PKB, thereby activating Akt/PKB (Alessi et al., 1996; Alessi et al., 1997). The activated Akt/PKB triggers many cellular responses, such as regulation of cell survival (Datta et al., 1997; del Peso et al., 1997), activation of glycogen synthesis (Shaw et al., 1997), stimulation of glucose uptake and translocation of glucose transporter 4 (Kohn et al., 1996), which leads to cell transformation (Marte et al., 1997), and induction of E2F transcriptional activity (Brennan et al., 1997). All of the Akt/PKB isoforms must be phosphorylated at the conserved threonine and serine residues for full activation (Thomas et al., 2002). Various physiological studies on Akt/PKB isoforms have revealed that Akt1/PKBα affects growth, neonatal mortality, and adipogenesis (Cho et al., 2001b; Garofalo et al., 2003; Yang et al., 2003), whereas Akt2/PKBβ regulates glucose metabolism (Cho et al., 2001a; Garofalo et al., 2003), and Akt3/PKBγ regulates brain size (Easton et al., 2005).

Recently, we reported that 8-Cl-cAMP and AICAR inhibited the growth of cancer cells through the activation of AMPK and p38 MAPK (Han et al., 2009). Moreover, other research groups also showed that activation of AMPK and p38 MAPK induces the growth inhibition of various cancer cells (Lucchi et al., 2011). It is well known that Akt/PKB is involved in cell survival by inhibiting apoptotic cell death. For this reason, we anticipated that the phosphorylation (activation) of Akt/PKB would be decreased during 8-Cl-cAMP-mediated growth inhibition. However, it turned out that treatment with 8Cl-cAMP and AICAR increased the phosphorylation of Akt/PKB. In addition, a recent report showed that various signaling pathways were able to activate both the Akt/PKB and AMPK (Brazil and Hemmings, 2001; Horie et al., 2008). Based on these new studies, we hypothesized that Akt/PKB might be involved in the activation of AMPK and p38 MAPK during 8-Cl-cAMP- and AICAR-induced inhibition of cancer cell growth. We found that 8-Cl-cAMP and AICAR indeed stimulated the phosphorylation of Akt2/PKBβ and that Akt2/PKBβ acted on AMPK and p38 MAPK as the upstream factor.

Materials and Methods

Reagents and antibodies

8-Cl-cAMP was purchased from Biolog (Bremen, Germany). AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) was obtained from Toronto Research Chemicals (Ontario, Canada). Triciribine (TCN), Compound C, and ABT702 were from Calbiochem (San Diego, CA). SB203580 was obtained from A. G. Scientific (San Diego, CA). Staurosporine (STSP), Annexin-V-FITC, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), A134974, and NBTI were purchased from Sigma-Aldrich (St. Louis, MO). Propidium iodide (PI) was from MP Biomedicals (Santa Ana, CA). GFP, Akt-1, and Akt-2 siRNA were produced by ST Pharm (Seoul, Korea). The NT sequence of the GFP siRNA was as follows: sense—GUU CAG CGU GUC CGG CGA GdTdT; antisense—CUC GCC GGA CAC GCU GAA CdTdT. The sequence of the Akt-1 siRNA was as follows: sense—GGA CAG AGG AGC AAG GUU UdTdT; antisense—AAA CCU UGC UCC UCU GUC CdTdT. The sequence of the Akt-2 siRNA was as follows: sense—CGA CUG AGG AGA UGG AAG UdTdT; antisense—ACU UCC AUC UCC UCA GUC GdTdT. The transfection reagent, Lipofectamine™ 2000, was from Invitrogen (Carlsbad, CA). The dominant negative expression vector with a kinase dead (KD) form of AMPKα2 was a kind gift from Dr. Morris J. Birnbaum (Howard Hughes Medical Institute, University of Pennsylvania Medical School, Philadelphia, PA) (Mu et al., 2001).

Phospho-Akt1/2/3 (Ser473), phospho-AMPKα (Thr172), total-Akt1/2/3, and total-AMPKα antibodies were purchased from Cell Signaling Technology (Danvers, MA). Phospho-mTOR (Ser2448), total-mTOR, and phospho-p38 (Thr180/Tyr182) MAPK antibodies were also obtained from Cell Signaling Technology. The total p38 MAPK antibody was from BioVision (Mountain View, CA), and the antibody for c-myc was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

HeLa (human cervical carcinoma) and MCF7 (human breast carcinoma) cells were maintained in Eagle's minimal essential medium (EMEM) (Hyclone, Logan, UT). SW-480 (human colon adenocarcinoma) and DLD1 (human colon adenocarcinoma) cells were cultured in RPMI1640 medium (Mediatech, Manassas, VA). Human dermal fibroblast (HDF) cells were cultured in DMEM medium (Hyclone). The EMEM and RPMI1640 were supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin G (A.G. Scientific, San Diego, CA), and 100 μg/ml streptomycin (Sigma-Aldrich, St.Louis, MO). The DMEM was supplemented with 15% fetal bovine serum (Hyclone). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cell counts and viability were determined using a Coulter™ counter (Beckman Coulter, Fullerton, CA) and an MTT assay, respectively. To measure cell death, cells were stained with PI (8 μg/ml, 25 min), and observed under an Optiphot-2 fluorescence microscope (Nikon, Tokyo, Japan).

Construction of stable cell line

The KD-AMPK (a kinase-dead form of AMPKα2) expression vector and pcDNA3.0 (mock) vector were transfected into HeLa cells using Lipofectamine™ 2000 according to the manufacturer's protocol. Successfully transfected cells were then selected in the presence of the antibiotic G418 (1 mg/ml) for 3 weeks.

MTT (3-[4,5-dimethylthiazol-2-yl]-diphenyl-tetrazolium bromide) assay

Five thousand cells were seeded into flat-bottomed 24-well plates. At an appropriate time interval, MTT (2 mg/ml in phosphate-buffered saline [PBS]) was added to the cultures. After incubating for 4 h, the resulting color reaction product, MTT formazan, was dissolved with dimethyl sulfoxide, and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA).

Western blot analysis

Western blot analysis was performed according to our previous publication (Han et al., 2009).

Transfection of siRNA

One day before transfection, 3×10^5 HeLa cells were seeded onto 60 mm culture dishes in EMEM without antibiotics. The cells were 30–50% confluent at the time of transfection. Akt-1, Akt-2, and GFP siRNAs were transfected into cells using Lipofectamine™ 2000 according to the manufacturer's protocol. Short interfering RNA-transfected HeLa cells were used for experiments after 24 h.

Results

Phosphorylation of Akt/PKB was increased upon treatment with 8-Cl-cAMP and AICAR

8-Cl-cAMP has been investigated as a potential anti-cancer drug, which was found to inhibit cellular growth and promote apoptosis in various cancer cell lines (Ciardiello et al., 1990; Kim et al., 2001). Treatment with 8-Cl-cAMP and AICAR was expected to decrease the phosphorylation/activation of Akt/PKB, because Akt/PKB was known to act as a survival signal in many cells. However, our results showed that treatment of

8-Cl-cAMP increased the phosphorylation of Akt/PKB in HeLa cells.

First, we tested whether the phosphorylation of Akt/PKB was altered during 8-Cl-cAMP-mediated growth inhibition in HeLa cells. HeLa cells were incubated with 8-Cl-cAMP (10 μM) for 3 days, and Western blotting was performed to measure the phosphorylation of Akt/PKB using antibodies against phospho-Akt1/2/3 and total Akt1/2/3 (Fig. 1A). All Akt/PKB isoforms were known to be phosphorylated at their conserved threonine and serine residues for full activation (Thomas et al., 2002). We found that the phosphorylation of Akt/PKB increased from 24 to 48 h in a time-dependent manner after treatment with 8-Cl-cAMP. Because we previously showed that AICAR shared the same pathway as 8-Cl-cAMP in inhibiting cancer cell growth (through AMPK and p38 MAPK activation) (Han et al., 2009), we also tested the effect of AICAR on Akt/PKB phosphorylation. When HeLa cells were incubated with AICAR for 6 h, the phosphorylation of Akt/PKB was increased in a time-dependent manner (Fig. 1B). As previously reported, the phosphorylation of both the AMPK and p38 MAPK was increased upon treatments with 8-Cl-cAMP and AICAR (Fig. 1A and B). The treatment with 8-Cl-Adenosine (10 μM), a metabolite of 8-Cl-cAMP, also stimulated the phosphorylation of Akt/PKB, AMPK and p38 MAPK just as 8-Cl-cAMP did (Fig. 1C).

To determine whether the phosphorylation of Akt/PKB by 8-Cl-cAMP was unique to HeLa cells, we also tested other cancer cell lines, such as SW480 (human colon cancer), DLD1 (human colon cancer), and MCF7 (human breast cancer). We found that 8-Cl-cAMP did promote the phosphorylation of Akt/PKB in all the cancer cell lines tested (Fig. 1D).

To confirm that the phosphorylation of Akt/PKB was a result of 8-Cl-cAMP or AICAR treatment, HeLa cells were pre-treated with 5-(p-nitrobenzyl)-6-thio-inosine (NBTI, an adenosine transporter inhibitor) and ABT702 (an adenosine kinase inhibitor) for 1 h before the treatment with 8-Cl-cAMP. The phosphorylation of Akt/PKB, AMPK, and p38 MAPK by AICAR and 8-Cl-cAMP was effectively attenuated by pre-treatment with NBTI and ABT702 (Fig. 2A–D). Furthermore, the phosphorylation of mTOR, a known downstream effector of Akt/PKB, was also decreased upon treatment with NBTI (Fig. 2C).

These data collectively show that treatment with 8-Cl-cAMP or AICAR induced the phosphorylation and activation of Akt/PKB in various cancer cell lines. Furthermore, to exert an effect on the phosphorylation and kinase activity of Akt/PKB, 8-Cl-cAMP and AICAR must be admitted into the cell via an adenosine transporter and then converted to their metabolites.

The anti-proliferative activity of 8-Cl-cAMP is limited to cancer cells

It has been claimed that 8-Cl-cAMP induced growth inhibition and/or apoptosis in cancer cells, whereas it has almost no effect in non-transformed counterparts (Ciardiello et al., 1990; Kim et al., 2001). We, therefore, decided to check once more if treatment of 8-Cl-cAMP is able to increase the phosphorylation of Akt/PKB, AMPK, and p38 MAPK in non-transformed cells. We employed HDF cells as non-transformed cell model and the effect of 8-Cl-cAMP in growth inhibition and cell death were monitored by cell counting, PI staining and microscopic analysis.

While 8-Cl-cAMP induced the phosphorylation of Akt/PKB, AMPK, and p38 MAPK in HeLa cells, it does not increase the phosphorylation of the same signaling molecules in HDF cells (Fig. 3A). Also, while the proliferation of HeLa cells was attenuated by 8-Cl-cAMP treatment, it did not show any inhibitory effect on the growth of HDF cells (Fig. 3B). In non-transformed HDF cells, 8-Cl-cAMP could not induce any remarkable cell death (PI-positive cells), either (Fig. 3C).

These data show that the treatment of 8-Cl-cAMP increased the phosphorylation of Akt/PKB, AMPK and p38 MAPK only in transformed cells, and that the anti-proliferative activity of 8-Cl-cAMP is limited to cancer or transformed cell lines.

Akt/PKB activates AMPK and p38 MAPK during 8-Cl-cAMP- or AICAR-induced growth inhibition

We previously demonstrated that the activation of AMPK and p38 MAPK upon treatment with 8-Cl-cAMP played a critical role during 8-Cl-cAMP-induced growth inhibition (Han et al., 2009). Accordingly, we investigated whether the phosphorylation of Akt/PKB was involved in the activation of AMPK and p38 MAPK during 8-Cl-cAMP- and AICAR-induced growth inhibition.

HeLa cells were treated with AICAR and 8-Cl-cAMP after 1 h of pre-treatment with Triciribine (TCN, an Akt1/2/3-specific inhibitor) (Fig. 4A and B). The AICAR-induced phosphorylation of Akt/PKB was reduced upon pre-treatment with TCN. The phosphorylation of AMPK and p38 MAPK was also decreased upon pre-treatment with TCN. The phosphorylation of mTOR was also abrogated by pre-treatment with TCN, indicating that the phosphorylation and activation of Akt/PKB was effectively blocked by TCN treatment (Fig. 4A). Similar to AICAR, co-treatment of TCN with 8-Cl-cAMP abolished the phosphorylation of Akt/PKB, AMPK, p38 MAPK, and mTOR in HeLa cells (Fig. 4B).

We also checked the involvement of PI3K in the activation of Akt/PKB during the 8-Cl-cAMP induced growth inhibition. Interestingly, while Akt-specific inhibitor, TCN, could attenuate AICAR or 8-Cl-cAMP-induced phosphorylation of AMPK and p38 MAPK (Fig. 4A and B), LY294002, a known PI3K inhibitor, could not inhibit the phosphorylation of these signaling molecules (Fig. 4C). Also, 8-Cl-cAMP and AICAR-induced cancer cell growth inhibition were not attenuated by LY294002 treatment (data not shown). These results indicate that PI3K, a known upstream effector of Akt/PKB, is not involved in the phosphorylation of Akt/PKB, AMPK, and p38 MAPK and in the 8-Cl-cAMP induced growth inhibition of HeLa cells.

These data collectively suggest that the phosphorylation of Akt/PKB occurred before the activation of AMPK and p38 MAPK, and that the activation of AMPK and p38 MAPK were dependent upon the activity of Akt/PKB during 8-Cl-cAMP- and AICAR-induced growth inhibition.

To confirm the involvement of the Akt/PKB kinase in 8-Cl-cAMP-induced growth inhibition, we employed siRNAs targeted against Akt/PKB. We constructed two different siRNAs against Akt/PKB. One was targeted against Akt1/PKBα (Akt-1 siRNA), and the other was targeted against Akt2/PKBβ (Akt-2 siRNA) (Fig. 5A). Using Western blot analysis, we were able to show that the level of Akt/PKB was significantly reduced 24 h after transfection with the Akt-1 and Akt-2 siRNAs (Fig. 5B). We were able to show by employing Akt-1, Akt-2, and Akt-3 specific antibodies that Akt-1 siRNA lowered the level of only Akt-1 protein, whereas Akt-2 siRNA lowered only Akt-2 protein level (Fig. 5C). We then tested whether the phosphorylation of AMPK and p38 MAPK upon treatment with 8-Cl-cAMP was affected by transfection with Akt-1 and Akt-2 siRNAs. As a control, we found that a GFP-targeted siRNA did not block the phosphorylation of AMPK and p38 MAPK in HeLa cells when exposed to 8-Cl-cAMP (Fig. 5C). When HeLa cells were transfected with Akt-2 siRNA, the phosphorylation of AMPK and p38 MAPK was significantly reduced, whereas transfection with Akt-1 siRNA did not influence the phosphorylation of AMPK, p38 MAPK, markedly (Fig. 5C).

These results demonstrated that among the three Akt/PKB isoforms, only Akt2/PKBβ appeared to be involved in the

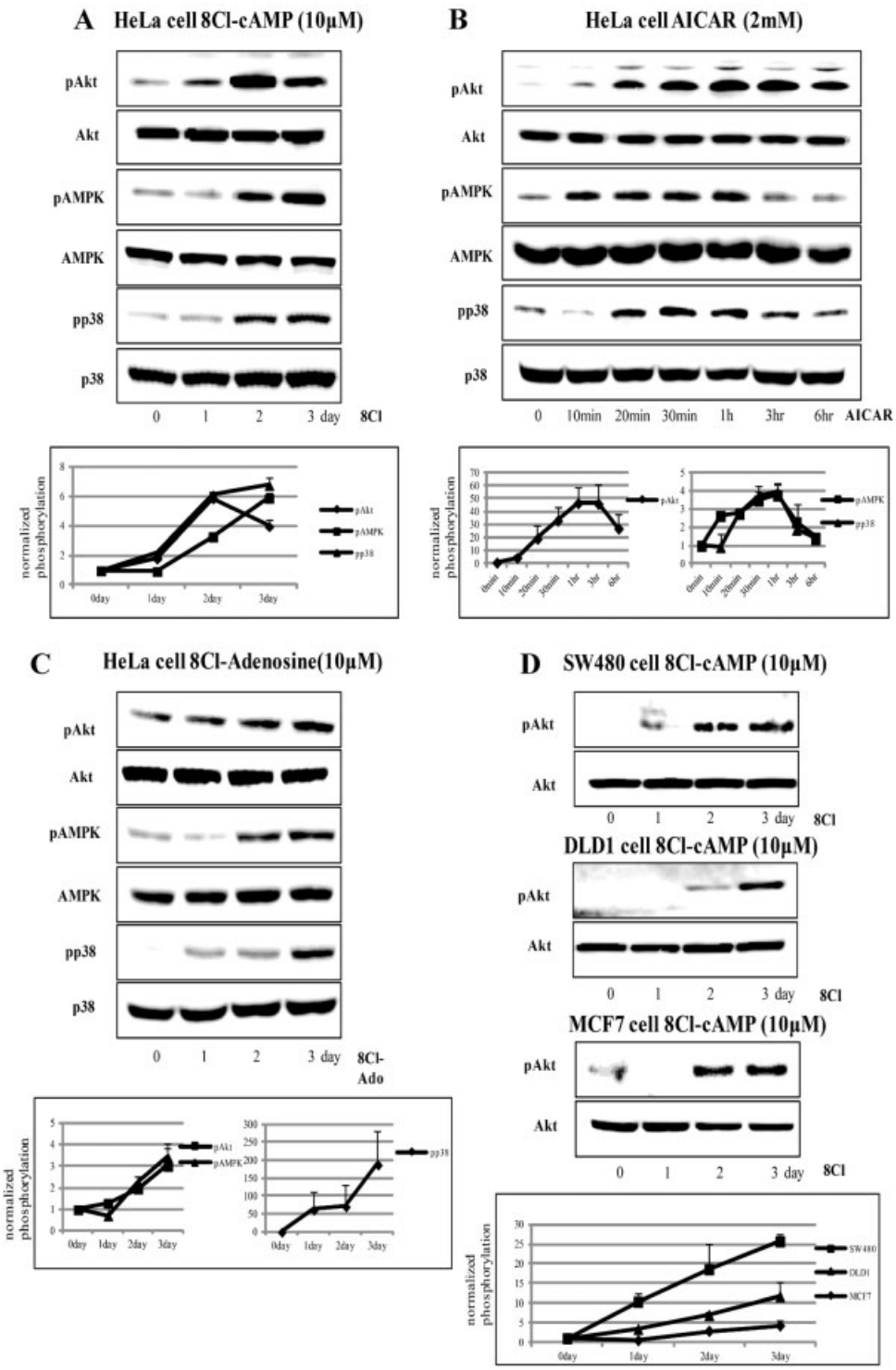

Fig. 1. Akt/PKB activity was increased after 8-Cl-cAMP and AICAR treatment in various cell lines. A and B: HeLa cells were treated with 8-Cl-cAMP (10 μmol/L) and AICAR (2 mmol/L) for the indicated times, and Western blotting was carried out using anti-phospho-Akt (pAkt), anti-Akt (Akt), anti-phospho-AMPK (pAMPK), anti-AMPK (AMPK), anti-phospho-p38 MAPK (pp38), and anti-p38 MAPK (p38) antibodies. C: HeLa cells were treated with 8-Cl-adenosine (10 μmol/L) for the indicated times, and Western blot was performed as in (A). D: SW480, DLD1, and MCF7 cells were treated with 8-Cl-cAMP (10 μmol/L) for the indicated times and Western blotting was performed using anti-phospho-Akt (pAkt) and anti-Akt (Akt) antibodies. The immunoblot signals were normalized by Multi-gauge™ ver.3.0 (FujiFilm, Tokyo, Japan). Graphs are mean ± SD value of phosphorylated protein/total protein intensity from three independent experiments.

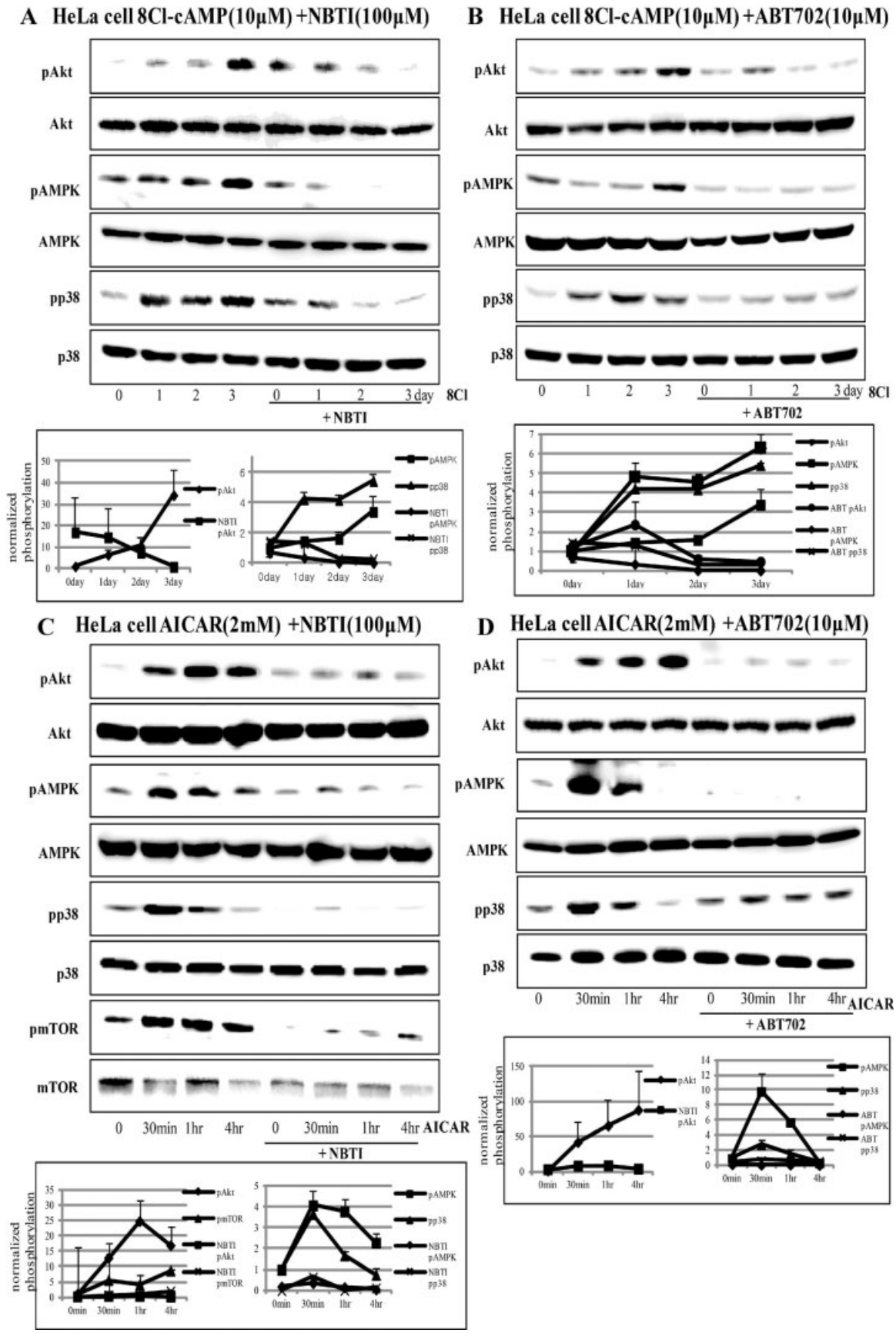

Fig. 2. 8-Cl-cAMP and AICAR treatment resulted in Akt/PKB phosphorylation. A–D: HeLa cells were treated with 8-Cl-cAMP and AICAR for the indicated times in the presence or absence of ABT702 (an adenosine kinase inhibitor, 10 μmol/L) and NBTI (an adenosine transporter inhibitor, 100 μmol/L). Western blotting was carried out using anti-phospho-Akt (pAkt), anti-Akt (Akt), anti-phospho-AMPK (pAMPK), anti-AMPK (AMPK), anti-phospho-p38 MAPK (pp38), anti-p38 MAPK (p38), anti-phospho-mTOR (pmTOR), and anti-mTOR (mTOR) antibodies. The immunoblot signals were normalized by Multi-gauge™ ver.3.0 (Fuji Film). Graphs are mean ± SD value of phosphorylated protein/total protein intensity from three independent experiments.

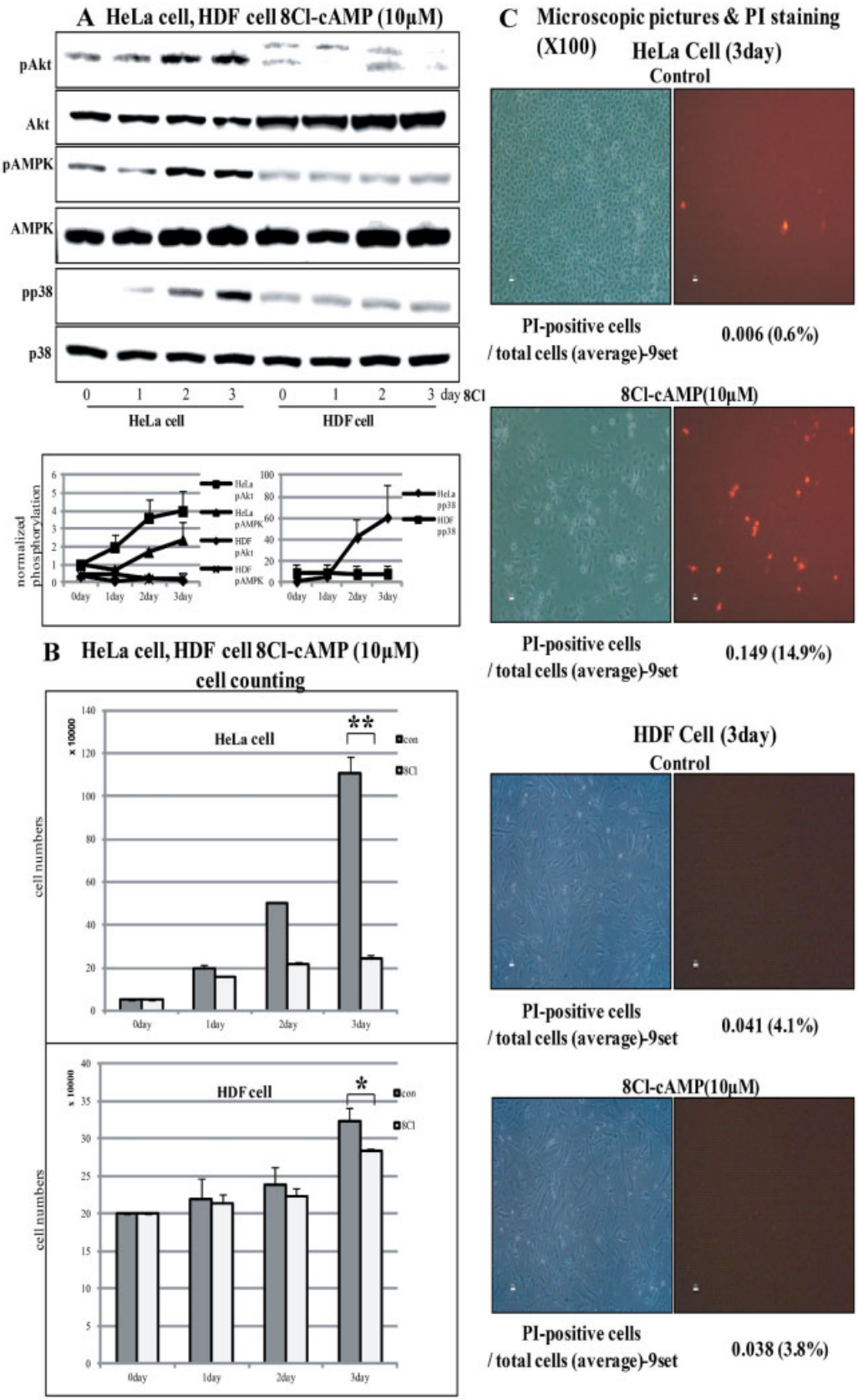

Fig. 3. The anti-proliferative activity of 8-Cl-cAMP is limited to cancer cells. A: HeLa and HDF cells were treated with 8-Cl-cAMP for the indicated times. Western blotting was carried out using anti-phospho-Akt (pAkt), anti-Akt (Akt), anti-phospho-AMPK (pAMPK), anti-AMPK (AMPK), anti-phospho-p38 MAPK (pp38), and anti-p38 MAPK (p38) antibodies. B: HeLa and HDF cells were incubated with 8-Cl-cAMP (10 μmol/L) for 3 days and cells were counted using a Coulter™ counter. Bars denote cell number (mean ± SD, n = 9). Asterisks indicate a significant difference from the control (*$P < 0.05$, **$P < 0.01$, two-tailed *t*-test). C: HeLa and HDF cells were incubated with 8-Cl-cAMP (10 μmol/L) for 3 days, and then PI staining (8 μg/ml, 25 min) was performed to visualize dead cells. Pictures were taken at ×100 magnification. The immunoblot signals were normalized by Multi-gauge™ ver.3.0 (Fuji Film). Graphs are mean ± SD value of phosphorylated protein/total protein intensity from three independent experiments.

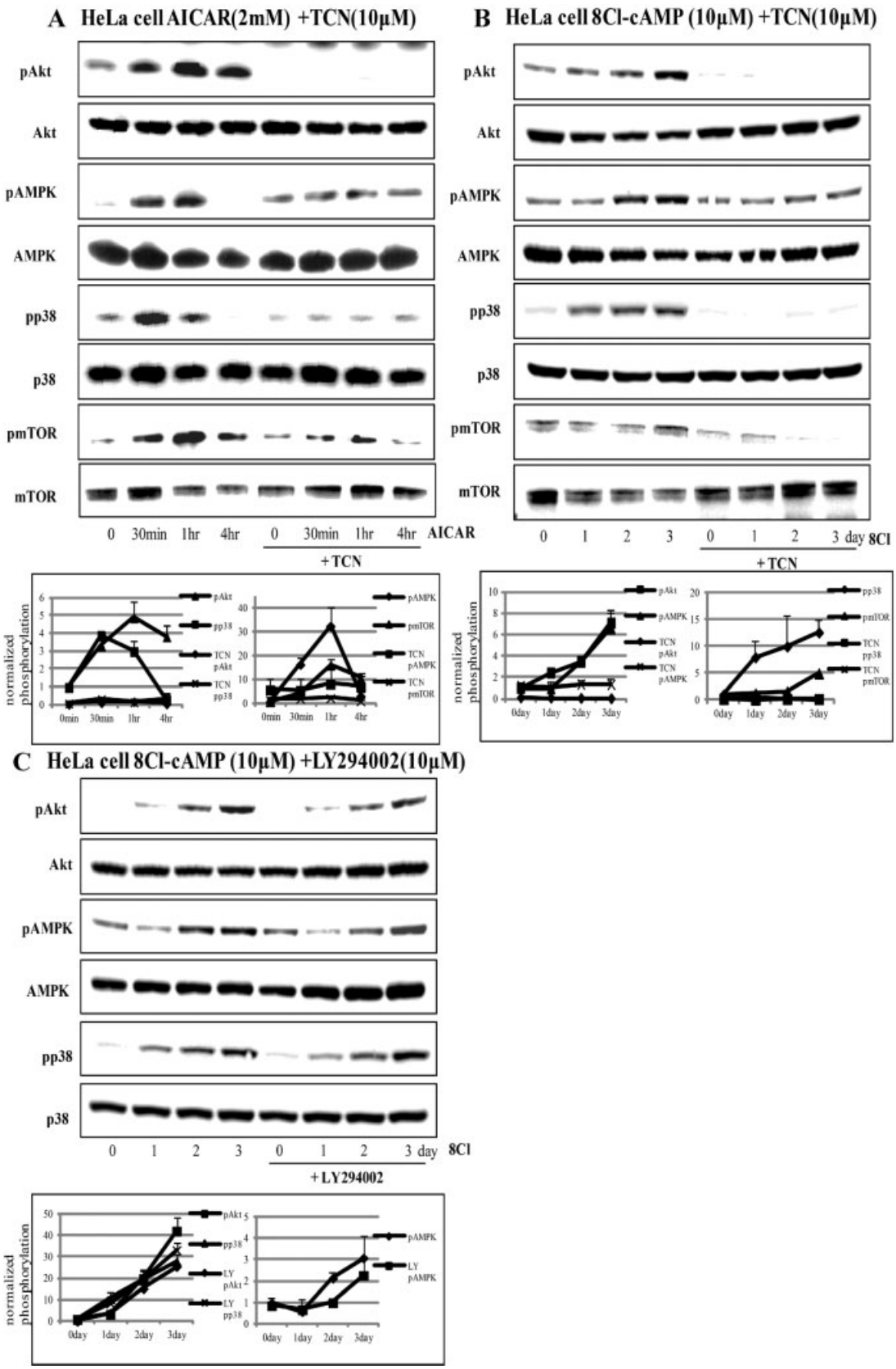

Fig. 4. Akt/PKB activated AMPK and p38 MAPK. A: HeLa cells were incubated with TCN (an Akt1/2/3 specific inhibitor, 10 μmol/L) for 1 h prior to AICAR treatment (2 mmol/L) for 4 h. Akt/PKB, AMPK, and p38 MAPK activation was assessed by Western blot with phospho-specific antibodies as in Figure 1. The decreases in mTOR phosphorylation upon TCN treatment indicated that the activation of Akt/PKB was attenuated by TCN. B: HeLa cells were incubated with 8-Cl-cAMP (10 μmol/L) for 1, 2, and 3 days with or without TCN (10 μmol/L). Western blotting was performed as in (A). C: HeLa cells were pre-treated with LY294002 (a PI3K specific inhibitor, 10 μmol/L) for 1 h prior to 8-Cl-cAMP (10 μmol/L) treatments. Western blotting was performed as in (A). The immunoblot signals were normalized by Multi-gauge™ ver.3.0 (Fuji Film). Graphs are mean ± SD value of phosphorylated protein/total protein intensity from three independent experiments.

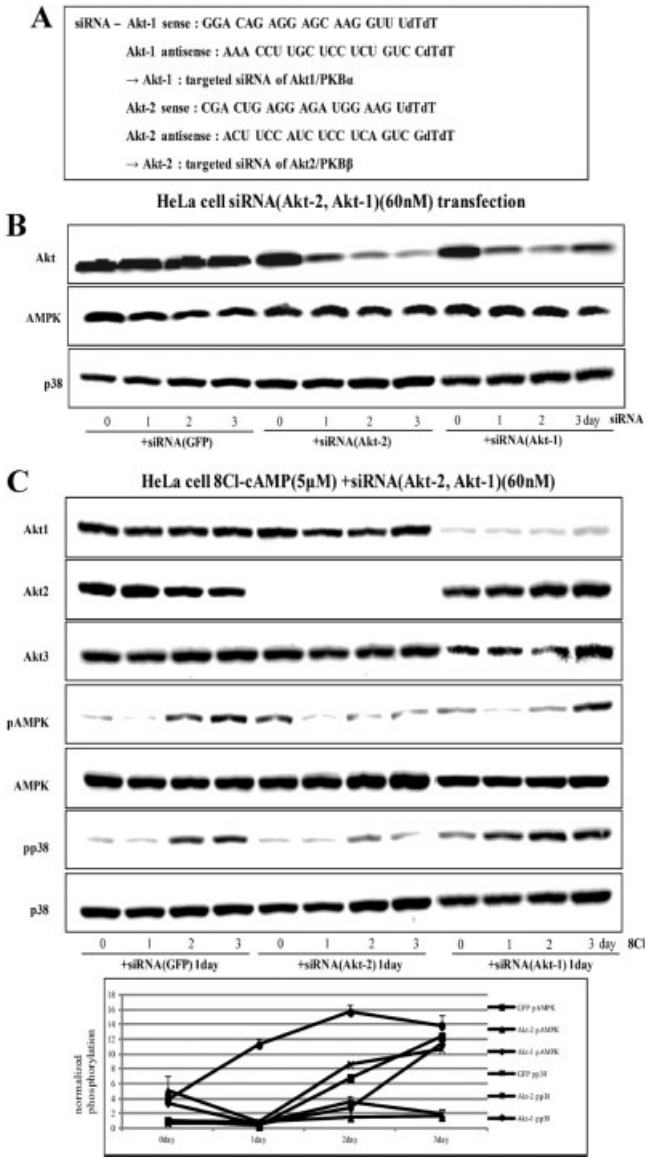

Fig. 5. siRNA targeted against Akt2/PKBβ attenuated the activation of AMPK and p38 MAPK upon treatment with 8-Cl-cAMP. A: The RNA sequences of the Akt-1/PKBα- and Akt-2/PKBβ-targeted siRNAs are shown. B: Akt-1-, Akt-2-, and GFP-targeted siRNAs were transfected into HeLa cells for the indicated times. C: HeLa cells were treated with 8-Cl-cAMP (5 μmol/L) for the indicated times 24 h after transfection with GFP-targeted siRNA (60 nmol/L), Akt2/PKBβ-targeted siRNA (60 nmol/L) and Akt1/PKBα-targeted siRNA (60 nmol/L). Western blotting was performed with anti-isoform specific-Akt (Akt1, Akt2, Akt3), anti-phospho-AMPK (pAMPK), anti-AMPK (AMPK), anti-phospho-p38 MAPK (pp38), and anti-p38 (p38) antibodies. The immunoblot signals were normalized by Multi-gauge™ ver.3.0 (Fuji Film). Graphs are mean ± SD value of phosphorylated protein/total protein intensity from three independent experiments.

phosphorylation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells induced by 8-Cl-cAMP.

The Akt1/2/3 specific inhibitor, TCN, blocked the growth inhibition and cell death mediated by 8-Cl-cAMP

Several cancer cell lines were employed in MTT assays as well as growth analyses to determine whether TCN could reverse the growth inhibitory effect of 8-Cl-cAMP. The 8-Cl-cAMP-induced growth inhibition was attenuated by co-treatment with TCN as determined by MTT assay (Fig. 6A) and cell counting (Fig. 6B–D) analysis. These results demonstrate that the growth inhibition induced by 8-Cl-cAMP treatment was mediated through the activation of Akt/PKB in various cancer cell lines.

The final approach to confirm the involvement of Akt/PKB in 8-Cl-cAMP-mediated growth inhibition in cancer cells was to examine the apoptotic signature. As shown in Figure 7A, the average number of PI-positive (dead) cells induced by 8-Cl-cAMP treatment was decreased upon co-treatment with TCN. We then used Annexin-V-FITC FACS analysis and DNA fragmentation analysis to determine whether PI-positive dead cells are apoptotic cells or necrotic cells (Fig. 7B and C). Annexin-V-FITC FACS analysis and DNA fragmentation data showed that apoptosis is not the major events during 8-Cl-cAMP-induced cancer cell death at least in HeLa cells (Fig. 7B and C). These data demonstrated that the inhibition of Akt2/PKBβ by TCN treatment attenuated the growth inhibition and cell death mediated by 8-Cl-cAMP treatment.

Akt2/PKBβ acts upstream of AMPK and p38 MAPK during 8-Cl-cAMP-induced growth inhibition

Thus far, we have shown that Akt2/PKBβ is responsible for the phosphorylation and activation of AMPK and p38 MAPK during 8-Cl-cAMP-mediated growth inhibition. To clarify the working order of these signaling molecules, we employed some chemical inhibitors as well as mutant construct. The selective AMPK inhibitor, Compound C, inhibited the phosphorylation of both p38 MAPK and MAPKAPK2 (a downstream effectors of p38 MAPK), whereas the phosphorylation of Akt2/PKBβ was not altered by treatment with Compound C during the 8-Cl-cAMP-induced inhibition of growth in HeLa cells (Fig. 8A). We use phospho-MAPKAPK2 antibodies for measuring kinase activity of p38 MAPK. To further study the effect of AMPK, we transfected AMPK-DN construct (a dominant negative kinase dead mutant of AMPK) into HeLa cells. In this construct, the lysine residue for ATP binding and hydrolysis was changed to arginine, resulting in the dominant negative phenotype of AMPK (Mu et al., 2001). The phosphorylation of p38 MAPK was decreased when AMPK-DN was over-expressed in cells treated with 8-Cl-cAMP, whereas the activation of Akt2/PKBβ was not affected (Fig. 8B). These data indicate that AMPK is working in between Akt2/PKBβ and p38 MAPK. In addition, the phosphorylation of MAPKAPK2 was blocked by SB203580, a p38 MAPK-specific inhibitor, but the phosphorylation of Akt2/PKBβ or AMPK was not affected in 8-Cl-cAMP-treated cells (Fig. 8C).

These results collectively demonstrate that Akt2/PKBβ acted upstream of AMPK and p38 MAPK is working downstream of AMPK during 8-Cl-cAMP-induced growth inhibition and cell death in HeLa cells.

Discussion

8-Cl-cAMP has been studied as a potent anti-cancer drug since it induces growth inhibition and apoptosis selectively in cancer cell lines while it has no effect on non-transformed cell lines (Ciardiello et al., 1990; Kim et al., 2001). However, the detailed mechanism of anti-cancer activity of 8-Cl-cAMP is still not fully understood.

Akt/PKB has been known to be involved in cell survival and proliferation as well as various signal transduction pathways. Especially, among the Akt/PKB isoforms, Akt2/PKBβ has been shown to regulate not only cell proliferation and survival (Huang et al., 2011; Chen et al., 2012; Fischer-Posovszky et al., 2012) but also macrophage function and density (Li et al., 2011), glucose uptake (Jensen et al., 2010), lipid synthesis and accumulation (Leavens et al., 2009), gastric acid secretion (Rotte et al., 2010), and osteoblast differentiation (Mukherjee

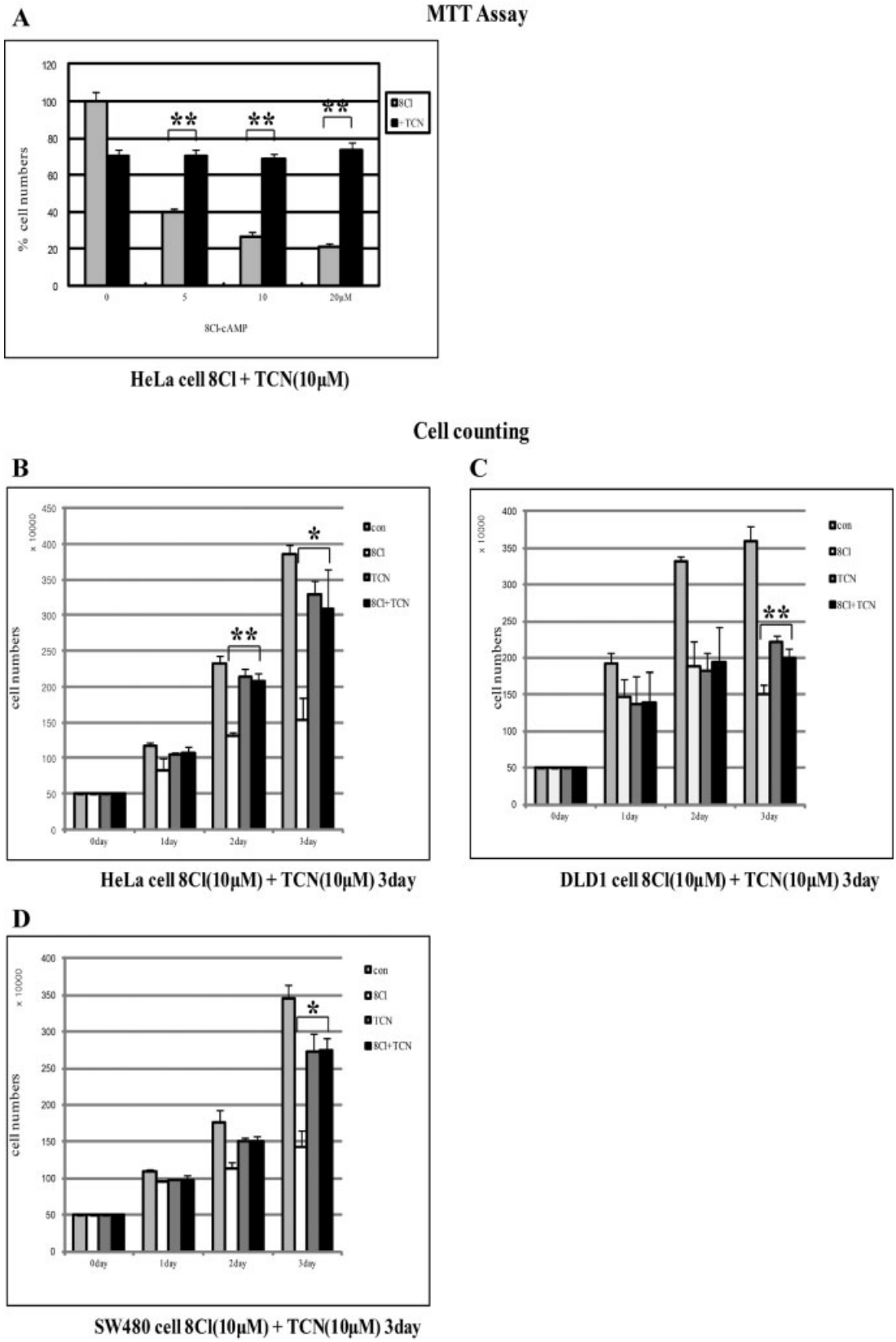

Fig. 6. The Akt-specific inhibitor, TCN, blocked 8-Cl-cAMP-induced growth inhibition. A: HeLa cells were incubated with 8-Cl-cAMP (0, 5, 10, and 20 μmol/L) for 3 days with or without TCN (10 μmol/L), and then the MTT assay was performed. Bars denote cell viability normalized to the control group (mean ± SD, n = 9). Asterisks indicate a significant difference from the control (*$P < 0.05$, **$P < 0.01$, two-tailed *t*-test). B–D: HeLa, DLD1 and SW480 cells were incubated with 8-Cl-cAMP (10 μmol/L) for 3 days with or without TCN (10 μmol/L), and cells were counted using a Coulter™ counter. Bars denote cell number (mean ± SD, n = 9). Asterisks indicate a significant difference from the control (*$P < 0.05$, **$P < 0.01$, two-tailed *t*-test).

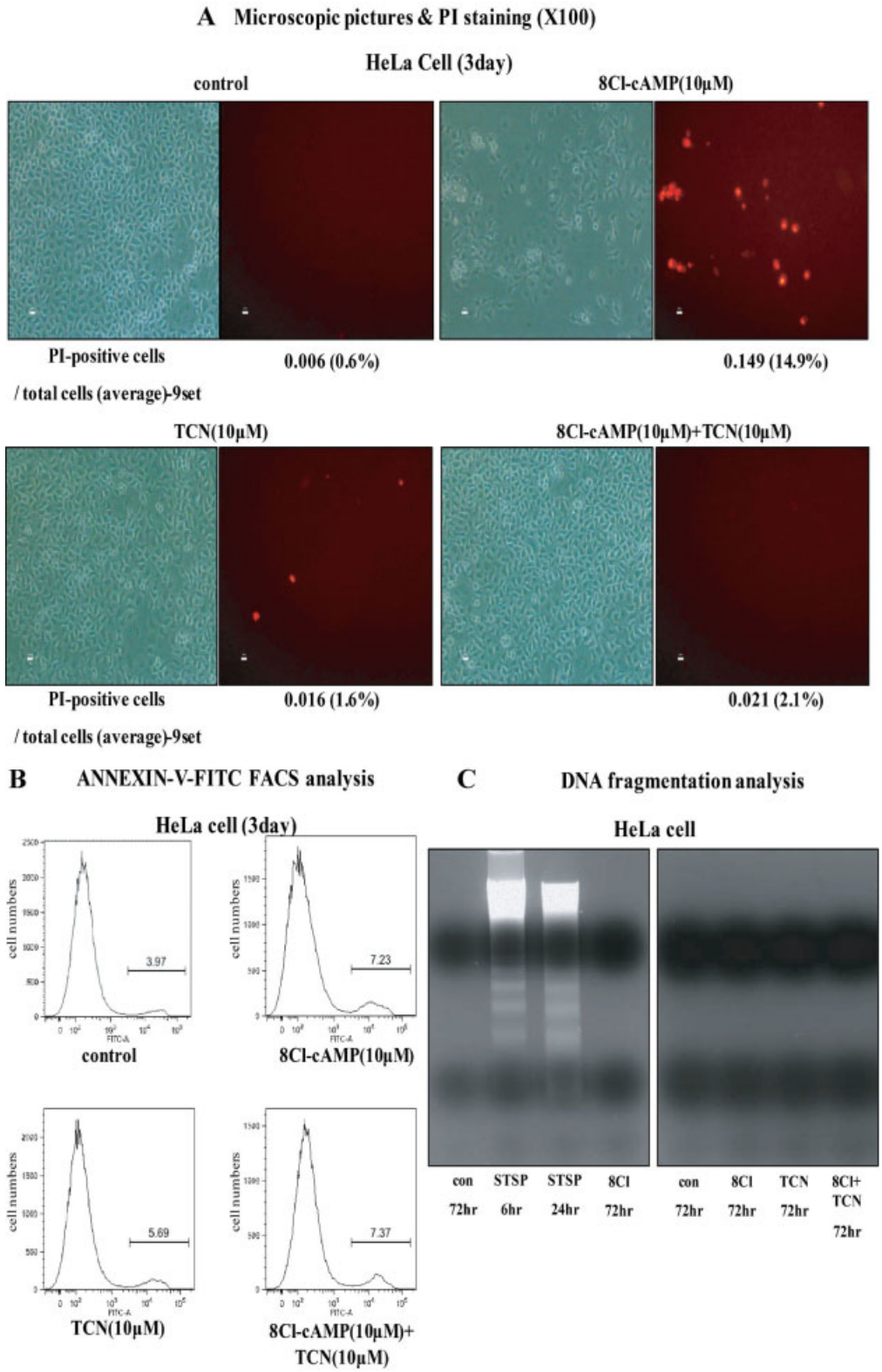

Fig. 7. TCN blocked cell death mediated by 8-Cl-cAMP treatments. A: The Akt-specific inhibitor reversed the 8-Cl-cAMP-induced growth inhibition and cell death. HeLa cells were incubated with 8-Cl-cAMP (10 μmol/L) for 3 days with or without TCN (10 μmol/L), and then PI staining (8 μg/ml, 25 min) was performed to visualize dead cells. Pictures were taken at ×100 magnification. B and C: HeLa cells were incubated with TCN (10 μmol/L) for 1 h prior to 8-Cl-cAMP treatment (10 μmol/L) for 3 days. 8-Cl-cAMP-induced apoptosis were measured using Annexin-V-FITC FACS analysis and DNA fragmentation analysis. Annexin-V-FITC analysis was performed using Annexin-V-FITC apoptosis detection kits (Sigma-Aldrich, St.Louis, MO) according to the manufacturer's protocol. The positive control was the groups of STSP (Staurosporine) treatment (1 μmol/L) for 6 and 24 h in DNA fragmentation analysis.

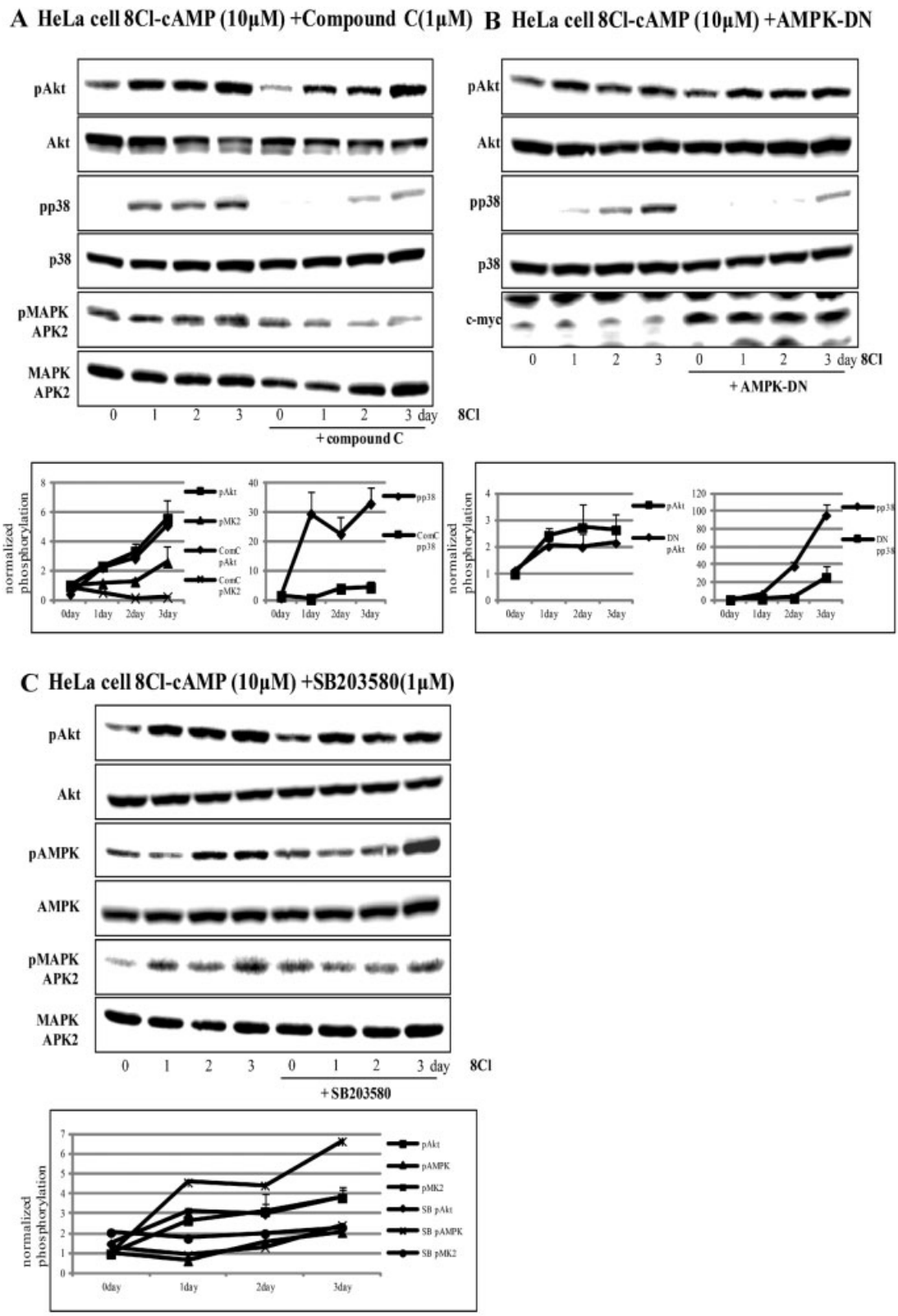

Fig. 8. Akt2/PKBβ acted upstream of AMPK and p38 MAPK during 8-Cl-cAMP- and 8-Cl-cAMP-induced growth inhibition. A: HeLa cells were pre-treated with Compound C (an AMPK specific inhibitor, 1 μmol/L) for 1 h prior to 8-Cl-cAMP (10 μmol/L) for 3 days. Western blotting was performed with anti-phospho-Akt (pAkt), anti-Akt (Akt), anti-phospho-p38 MAPK (pp38), anti-p38 (p38), anti-phospho-MAPKAPK2 (pMAPKAPK2 or pMK2), and anti-MAPKAPK2 (MAPKAPK2 or MK2) antibodies. B: The expression level of KD-AMPK-transfected stable cells (AMPK-DN) was confirmed by Western blotting using a c-myc antibody. HeLa and KD-AMPK-transfected cells were treated with 8-Cl-cAMP (10 μmol/L) for 3 days, and Akt/PKB and p38 MAPK activation was determined by Western blotting with anti-phospho-Akt (pAkt), anti-Akt (Akt), anti-phospho-p38 MAPK (pp38), anti-p38 (p38), and anti-c-myc (c-myc) antibodies. C: HeLa cells were pre-treated with SB203580 (a p38 MAPK specific inhibitor, 1 μmol/L) for 1 h prior to 8-Cl-cAMP (10 μmol/L) for 3 days. Western blotting was performed as in (A). The immunoblot signals were normalized by Multi-gauge™ ver.3.0 (Fuji Film). Graphs are mean ± SD value of phosphorylated protein/total protein intensity from three independent experiments.

et al., 2010). However, little has been reported about the involvement of Akt2/PKBβ in cancer cell growth inhibition.

We previously reported that the activation of AMPK and p38 MAPK inhibited cellular growth and induced cell death in various cancer cells during the treatment of 8-Cl-cAMP and AICAR (Han et al., 2009). In this report, we employed various experimental tools such as Western blot analysis, PI staining and cell counting analysis using specific inhibitors for Akt/PKB, AMPK, and p38 MAPK as well as Akt2/PKBβ-targeted siRNA. We showed that the activation of AMPK and p38 MAPK by the treatment of 8-Cl-cAMP and AICAR are actually mediated through the activation of Akt2/PKBβ. The data point to the direction that the activation of Akt2/PKBβ can act as one of the upstream trigger factors in 8-Cl-cAMP-induced cancer cell growth inhibition and cell death. We also showed in Figure 3 that the anti-proliferative activity of 8-Cl-cAMP is limited to cancer cells by employing non-transformed HDF cells and that the Akt/PKB as well as the downstream kinases AMPK and p38 MAPK are not activated by 8-Cl-cAMP treatment in this non-transformed cell line. This may serve as the clue for why 8-Cl-cAMP exerts its growth inhibitory effect only in the cancer cell lines and not in the non-transformed counterpart.

The use of specific inhibitors, that is Triciribine (TCN, an Akt1/2/3-specific inhibitor) and LY294002 (a PI3K inhibitor), clearly shows the direct involvement of Akt/PKB in the activation of AMPK and p38 MAPK. Though we are not presenting the data, Rapamycin, an mTOR (a known downstream effector of Akt/PKB) inhibitor, did not affect the phosphorylation of AMPK and p38 MAPK induced by the 8-Cl-cAMP treatment. LY294002 and Rapamycin treatment did not affect the overall cell growth inhibition induced by 8-Cl-cAMP treatment (data not shown), either.

The employment of Akt2/PKBβ-targeted siRNA clearly defined the involvement of Akt2/PKBβ but not Akt1/PKBα in the phosphorylation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells by 8-Cl-cAMP treatment. Overall, the data point to the direction that the cancer cell growth inhibition by 8-Cl-cAMP is mediated through Akt2/PKBβ signal transduction pathway, which seems to be PI3K-independent. In the conventional Akt/PKB signaling pathway, Akt/PKB is known to be phosphorylated at Thr308 and Ser473 residues by PDK1 and PDK2, which are activated by PI3K kinase (Alessi et al., 1996, 1997). In our case, treatment with 8-Cl-cAMP appeared to activate Akt2/PKBβ directly or through a PI3K-independent, unknown signaling pathway and mTOR is not involved in the 8-Cl-cAMP-mediated growth inhibition and cell death.

Up until now, Akt2/PKBβ has been known to be involved in cell proliferation and survival while it suppresses growth inhibition and apoptosis (Huang et al., 2011; Chen et al., 2012; Fischer-Posovszky et al., 2012). Our data in this report show unusual involvement of Akt2/PKBβ in that the treatment of anti-cancer drug induces Akt2/PKBβ activation and this activated Akt2/PKBβ is involved in the growth inhibition of cancer cells. Another research group reported relevant case that Akt2/PKBβ over-expression induced inhibition of cell proliferation through the up-regulation of the cell-cycle inhibitor p27 and inhibition of cyclin E/CDK2 complex in MDA-MD-231 breast cancer cells (Yang et al., 2011). We suggest that the activation of Akt2/PKBβ could induce cancer cell-specific growth inhibition and death depending on the particular cell type or anti-cancer drugs. If that is the case, Akt2/PKBβ can become the novel anti-cancer target for the control of cancer cell proliferation.

One remaining questions to be addressed is why the time course of 8-Cl-cAMP-induced phosphorylation of Akt2/PKBβ, AMPK, and p38 MAPK is different from AICAR-induced phosphorylation of the same molecules. So far, we do not have the correct answers. But our guess is that while AICAR activates Akt2/PKBβ and AMPK physically or directly, the action of 8-Cl-cAMP may be indirect. The report by Lucchi et al. (2011) showed that 8-Cl-cAMP and AICAR induced apoptosis of various cancer cells through the activation of AMPK and p38 MAPK. This paper also showed that while the activation of p38 MAPK by 8-Cl-cAMP took more than 24 h, the activation of Erk MAPK by 8-Cl-cAMP was achieved within 5 min of treatment. Hence, the existence of quick activation of Erk MAPK by 8-Cl-cAMP could support the possible existence of another signaling pathway for the activation of Akt2/PKBβ, AMPK, and p38 MAPK in the course of cancer cell growth inhibition.

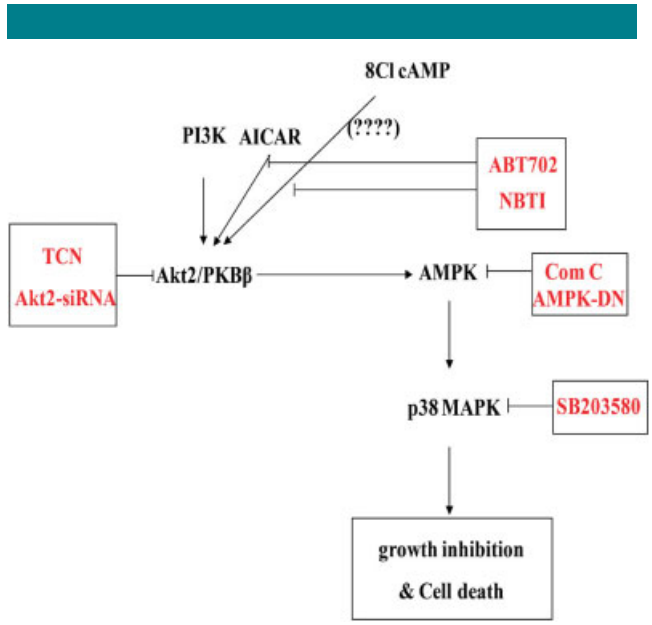

Fig. 9. Diagram showing the hierarchical working order of signaling molecules in 8-Cl-cAMP induced growth inhibition

In summary, we demonstrated that Akt2/PKBβ is involved in the regulation of 8-Cl-cAMP and AICAR-induced cancer cell growth inhibition and cell death through the activation of AMPK and p38 MAPK. Also, from the data presented in this report, we can clearly establish the order of action for the three signaling molecules involved, that is Akt/PKB working in the most upstream, AMPK in the middle and p38 MAPK at the downstream (Fig. 9).

Currently, attempts are being made to identify the missing intermediate molecules between the Akt2/PKBβ and/or AMPK and/or p38 MAPK in this 8-Cl-cAMP induced growth inhibition in cancer cells.

Acknowledgments

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