이학박사학위논문

8-Cl-cAMP가 유도하는 암세포의 세포성장억제 과정에서 작용하는 Akt2와 SHC1 단백질의 기능에 관한 연구

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2014년 2월

서울대학교 대학원

협동과정 유전공학전공

최 기 영

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Role of Akt2 and SHC1 proteins in 8-Cl-cAMP-induced cancer cell growth inhibition

A dissertation submitted in partial fulfillment

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To the Faculty of

Interdisciplinary Graduate Program in Genetic Engineering

at

Seoul National University

by

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ABSTRACT

Role of Akt2 and SHC1 proteins in 8-Cl-cAMP-induced cancer cell growth inhibition

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The Graduate School

Seoul National University

Cyclic AMP is a secondary messenger which plays a critical role(s) in various cellular physiological functions including regulation of cell growth and death. 8-chloro-cyclic AMP (8-Cl-cAMP) is one of the site-selective cAMP analogs that induce growth inhibition, apoptosis, differentiation and reverse transformation in a broad spectrum of cancer cell lines. Although 8-Cl-cAMP promotes tumor cell-specific growth inhibition and apoptosis, the exact signaling pathways for its action are still uncertain. Many research groups have made an effort to elucidate the signal pathways of 8-Cl-cAMP-induced cancer cell specific growth inhibition and

apoptosis. Through several studies, we knew that 8-Cl-cAMP promotes the down-regulation of RI subunit of PKA, the activation of protein kinase C (PKC), Rap1, AMP-activated protein kinase (AMPK) as well as the p38 mitogen activated protein kinase (p38 MAPK) during the growth inhibition or apoptosis of cancer cells. Also, it was confirmed that the conversion of 8-Cl-cAMP to 8-Cl-adenosine is prerequisite for its cell growth inhibitory effects.

In this thesis, I tried to show that Akt/protein kinase B (PKB) as well as Src homology 2 domain containing transforming protein 1 (SHC1) proteins were involved in 8-Cl-cAMP-induced cancer cell growth inhibition.

Akt/PKB genes encode three isoforms, Akt1, Akt2 and Akt3, which belong to the serine/threonine-specific protein kinase family. Akt/PKB protein has been known for its ability to confer cells to enhance cell survival by inhibiting apoptosis. Accordingly, it is expected that the activation or phosphorylation of Akt/PKB would be decreased upon treatment with 8-Cl-cAMP. Contrary to the expectations, however, the phosphorylation of Akt/PKB was increased by treatment with 8-Cl-cAMP. The increased phosphorylation of Akt/PKB was repressed by treatments of ABT702 (an adenosine kinase inhibitor) and NBTI (an adenosine transporter inhibitor). Furthermore, the 8-Cl-cAMP-induced phosphorylation of Akt/PKB was not attenuated by the treatments of Compound C (an AMPK inhibitor), AMPK-DN (AMPK-dominant negative) mutant and SB203580 (a p38 MAPK inhibitor), whereas TCN (an Akt1/2/3 specific inhibitor) and an *Akt2/PKBβ*-targeted siRNA

repressed the 8-Cl-cAMP- and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, an AMPK activator)-mediated phosphorylation of AMPK and p38 MAPK. TCN also restored the growth inhibitory effect mediated by 8-Cl-cAMP and AICAR, whereas, the *Akt1/PKBα*-targeted siRNA did not reduce the 8-Cl-cAMP-induced phosphorylation of AMPK and p38 MAPK. The obtained results pointed to the direction that the treatments of 8-Cl-cAMP and AICAR increased the phosphorylation of Akt2/PKBβ, which in turn stimulated the activation of AMPK and p38 MAPK.

SHC1 protein is expressed as three isoforms, 46, 52 and 66 kDa, which belong to the adaptor protein containing Src homology 2 (SH2) domains and each isoform has been shown to exert different functions in cellular response. In this thesis, it was shown that the treatment of 8-Cl-cAMP to cancer cells lower the phosphorylation level of SHC1, whereas the total amount of SHC1 protein was not altered. Furthermore, Compound C (an AMPK inhibitor) and SB203580 (a p38 MAPK inhibitor) did not block the 8-Cl-cAMP-induced decrease of SHC1 phosphorylation. However, when *SHC1*-targeted siRNAs were introduced in order to mimic the decrease of SHC1 phosphorylation, the decreased phosphorylaton and total amount of SHC1 protein seemed to stimulate the phosphorylation of AMPK and p38 MAPK similar to 8-Cl-cAMP treatment. These results suggest that reduced level of SHC1 phosphorylation acted upon the phosphorylation of AMPK and p38 MAPK as an upstream regulation factor during 8-Cl-cAMP-induced growth

inhibition.

Keywords : Akt/PKB, SHC1, 8-Cl-cAMP, cancer cell, growth inhibition

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TABLE OF CONTENTS

page

LIST OF FIGURES AND TABLES

page

INTRODUCTION

Anti-tumor agent is the general collective term for the chemotherapeutic drugs which possess the capability of cancer cell growth inhibition or death. It is believed that most important property of anti-tumor agent is the cancer cell specificity for its efficient anti-tumorigenic properties. The chemotherapeutic treatments with poor cancer specific anti-tumor agents could lead to severe side effects on patients. Therefore, to develop more cancer specific anti-tumor agents, it requires an accurate cancer cell-specific signal pathways related to cell growth, but not to normal cell growth. The cancer cell-specific growth signal pathways have been studied in our laboratory for a long time and some positive results were obtained (Ahn et al., 2006; Ahn et al., 2004; Ahn et al., 2005; Choi et al., 2013; Han et al., 2009; Kim et al., 1996; Kim et al., 2001; Kim et al., 2000a; Kim et al., 2000b; Kim et al., 1997; Lee et al., 1999).

The well-known secondary messenger cyclic AMP (cAMP) has been known to play a critical role(s) in the regulation of various cellular functions, such as cell differentiation (Cao et al., 2013; Yu et al., 2013), proliferation (Lyons et al., 2013; Steven et al., 2013), tumorigenesis and cancer development (Steven et al., 2013), transcriptional regulation of various genes (Sasi et al., 2013), and so on.

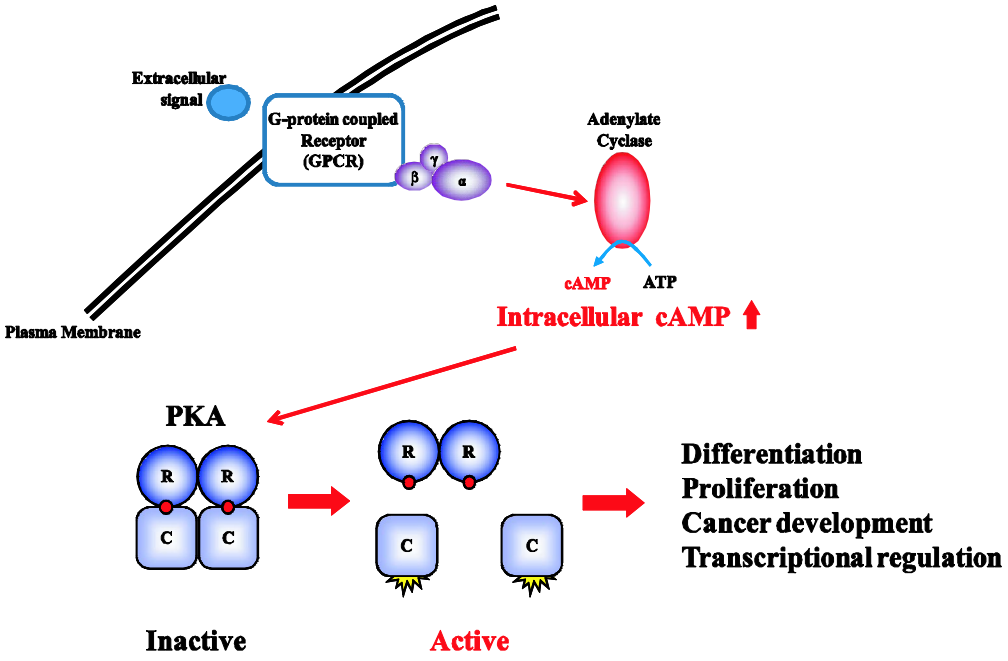
Extracellular signals such as insulin can stimulate the adenylate cyclases which convert ATP into cAMP, which in turn increased the level of intracellular. The cAMP-dependent protein kinase A (PKA), which has been known as the major target of cAMP, consists of the R subunit dimer and two C subunits. Cellular PKA is divided into two subtypes, type I and type II PKA, which are distinguished by which regulatory subunits (RI or RII) are bound with common catalytic subunit (C) (Cho-Chung et al., 1991; Kim et al., 2001). Generally speaking, cell proliferation and transformation were found to be more associated with high level of RI subunit expression, whereas cell growth inhibition and differentiation were correlated with down-regulation of RI subunit and/or up-regulation of RII subunit expression (Cho-Chung et al., 1991). Figure 1 shows the simplified diagram of cyclic AMP-PKA signaling pathway with regard to the various physiological conditions including the regulation of cellular growth.

8-Chloro-cyclic AMP (8-Cl-cAMP) is one of the site-selective cAMP analogs among many. 8-Cl-cAMP has been known to associate with the growth inhibition, apoptosis, reverse transformation and differentiation in various cancer cell lines (Kim et al., 2000a; Tagliaferri et al., 1988a; Tagliaferri et al., 1988b; Tortora et al., 1991). Whereas 8-Cl-cAMP is known to promote the growth inhibition and apoptosis in various cancer cells, it cannot inhibit the proliferation of non-transformed, normal cells (Choi et al., 2013; Ciardiello et al., 1990; Kim et al., 2001). Although 8-Cl-cAMP is expected to be an effective anti-tumor agent, the

Figure 1. The simplified diagram of cyclic AMP - PKA signal transduction pathway

Extracellular signals sequentially activate the adenylate cyclase which converts ATP into cAMP to increase the intracellular cAMP level. The increased cAMP is then bound to the regulatory subunits of the PKA, allowing the dissociation of the catalytic subunits from the holoenzyme. This separation from the regulatory subunits activates the catalytic subunits, which then look for the downstream substrates, mostly inside the nucleus, to complete the signaling pathway in controlling the cellular functions.

cyclicAMP-PKA signal transduction



type I PKA(RI) ↑ → cell proliferation, transformation

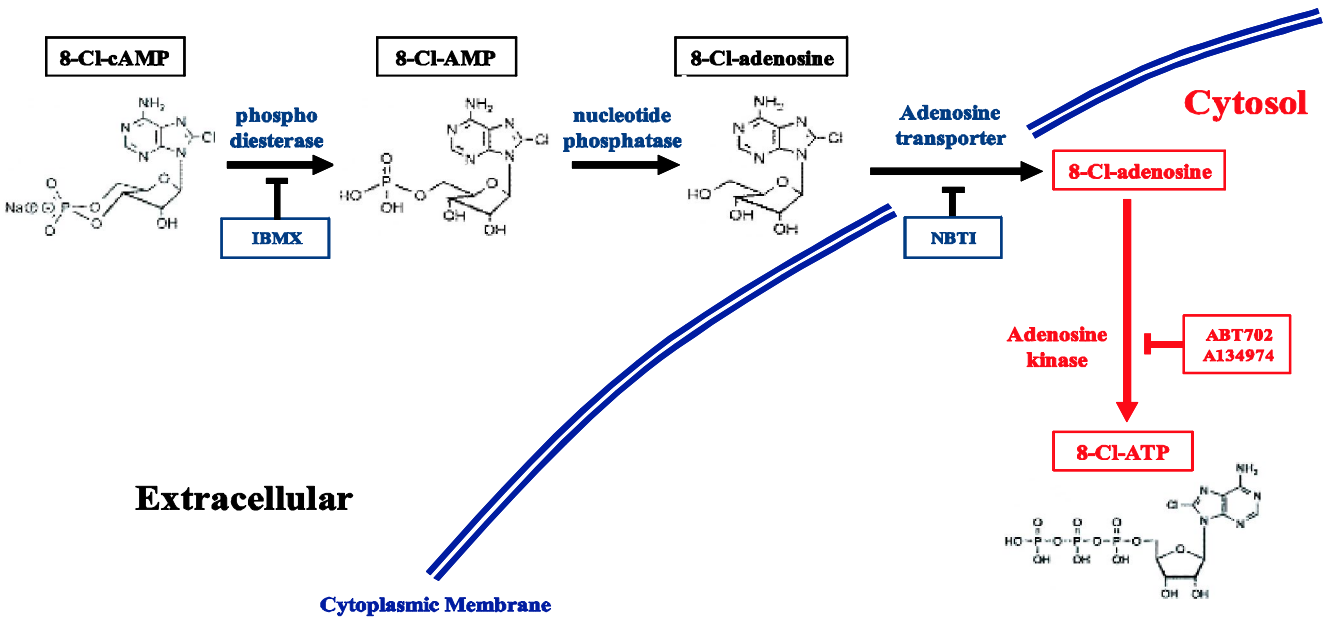
type I PKA(RI) ↓
type II PKA(RII) ↑ → cell growth inhibition, differentiation

accurate action mechanism(s) of 8-Cl-cAMP in controlling the cell proliferation are not fully understood, up to date.

8-Cl-cAMP is known to promote the down-regulation of RI subunit of PKA, which in turn mediate the inhibition of cell proliferation (Ciardiello et al., 1990; Kim et al., 2001). Also, the conversion of 8-Cl-cAMP to 8-Cl-adenosine, one of the metabolites of 8-Cl-cAMP, was proven to be an essential process for its cellular growth inhibitory effect (Cho-Chung, 1991; Lange-Carter et al., 1993). 8-Cl-cAMP is known to be converted into its various metabolites by sequential action of enzymes, such as phosphodiesterase (PDE), nucleotide phosphatase and adenosine kinase (Ahn et al., 2004; Han et al., 2009) (Fig. 2). The anti-proliferative effects of 8-Cl-cAMP, 8-Cl-AMP and 8-Cl-adenosine were interrupted by the treatment of adenosine deaminase which converts 8-Cl-adenosine to 8-Cl-inosine (Cho-Chung, 1991; Lange-Carter et al., 1993). Furthermore, 5-(p-nitrobenzyl)-6-thio-inosine (NBTI), an inhibitor of adenosine uptake process, and A134974, a selective adenosine kinase inhibitor, were shown to block the 8-Cl-cAMP-induced growth inhibition in cancer cells (Halgren et al., 1998). These studies indicated that the conversion of 8-Cl-cAMP to its metabolites is crucial to the inhibitory effects of cancer cell growth. Similar conclusions were drawn from the works of our laboratory that the conversion of 8-Cl-cAMP to 8-Cl-adenosine was indispensable for its growth inhibitory effects in many cancer cell lines (Ahn et al., 2004; Ahn et al., 2005; Han et al., 2009). In addition, we have studied various aspects of the

Figure 2. The conversion of 8-Cl-cAMP to its metabolites

8-Cl-cAMP is converted into its various metabolites by the activity of phosphodiesterase, nucleotide phosphatase and adenosine kinase. The adenosine transporter is known to allow 8-Cl-adenosine to enter the cytosol of the cells. Once inside the cell, 8-Cl-adenosine is known to be converted to 8-Cl-ATP to enforce the growth inhibitory effect (Ahn et al., 2004; Halgren et al., 1998). When 8-Cl-adenosine is converted to 8-Cl-inosine by adenosine deaminase, it was no longer effective in exerting growth inhibition in cancer cells.


8-Cl-cAMP
phospho diesterase
IBMX
8-Cl-AMP
nucleotide phosphatase
8-Cl-adenosine
Adenosine transporter
NBTI
Cytosol
8-Cl-adenosine
Adenosine kinase
ABT702
A134974
8-Cl-ATP
Extracellular
Cytoplasmic Membrane

signal transduction pathways of 8-Cl-cAMP-induced cancer cell-specific growth inhibition and apoptosis. It was shown that the regulation of cancer cell growth mediated by 8-Cl-cAMP and 8-Cl-adenosine was associated with the activation of protein kinase C (PKC), Rap1 and p38 mitogen activated protein kinase (p38 MAPK) in mouse fibroblast DT cells (Ahn et al., 2006; Ahn et al., 2004). It was also shown that the activation of AMP-activated protein kinase (AMPK) functioned upstream of p38 MAPK during the 8-Cl-cAMP-induced cancer cell growth inhibition in HeLa cells (Ahn et al., 2005; Han et al., 2009). The RI subunit of PKA was shown to be down-regulated through the activation of p38 MAPK in HL60 cells (Ahn et al., 2005). The plausible signaling pathway(s) involved in the 8-Cl-cAMP-induced cancer cell growth inhibition and apoptosis is depicted in Figure 3, which are summarized from the previous results obtained in our laboratory. Based on this figure, I tried in this thesis to fill up the missing links (or gaps) in analyzing the 8-Cl-cAMP induced cancer cell specific growth inhibitory effects.

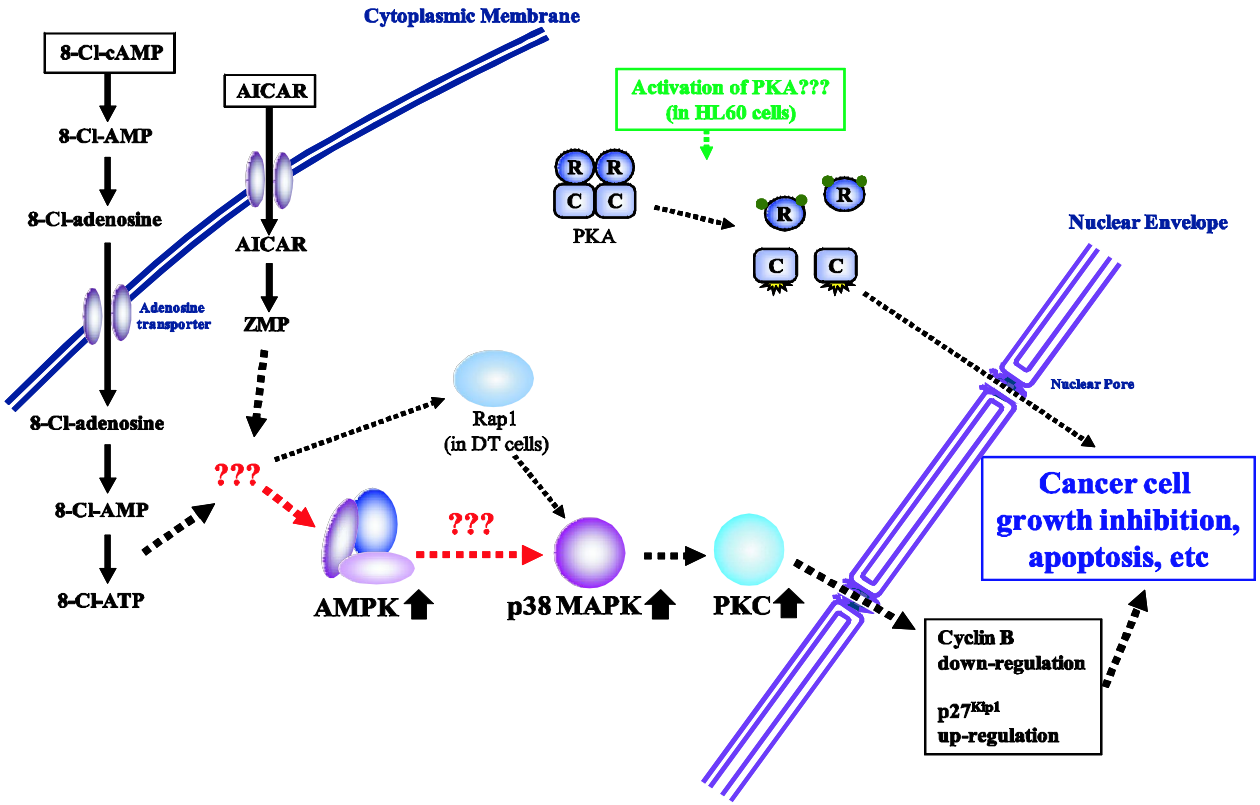
Akt/protein kinase B (PKB) was introduced as a serine/threonine-specific protein kinase in 1991 (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991b) and three very similar isoforms have been characterized so far. The first identified Akt/PKB was named Akt1/PKBα, the second one was named Akt2/PKBβ (Jones et al., 1991a) and the third one was designated Akt3/PKBγ (Konishi et al., 1995). Akt/PKB is known to be activated by various growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin,

Figure 3. Known signaling pathway(s) of 8-Cl-cAMP-induced cancer cell growth inhibition and apoptosis

This figure describes the PKA-dependent signaling pathway and the PKA-independent signaling pathway in 8-Cl-cAMP-induced cancer cell growth inhibition and apoptosis. The purpose of this study was to find the signaling molecules that are working around the AMPK and p38 MAPK (Red ??? in the figure).

AICAR: 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

ZMP: AICAR-5-monophosphate


Cytoplasmic Membrane
8-Cl-cAMP
8-Cl-AMP
8-Cl-adenosine
Adenosine transporter
8-Cl-adenosine
8-Cl-AMP
8-Cl-ATP
AICAR
AICAR
ZMP
???
Rap1
(in DT cells)
???
AMPK
p38 MAPK
PKC
Activation of PKA???
(in HL60 cells)
R
R
C
C
PKA
R
R
C
C
Nuclear Envelope
Nuclear Pore
Cancer cell growth inhibition, apoptosis, etc
Cyclin B down-regulation
p27Kip1 up-regulation

thrombin and nerve growth factor (NGF), etc (Downward, 1998). Phosphoinositide 3-kinase (PI3K) was known to modulate the activation of Akt/PKB upon the stimulation by several growth factors listed above (Burgering and Coffer, 1995; Franke et al., 1995). Akt/PKB was also known to be activated through the action of two protein kinases, phosphoinositide-dependent kinase 1 (PDK1) and phosphoinositide-dependent kinase 2 (PDK2). The activated PDK1 and PDK2 stimulated the addition of phosphates to Thr(308) and Ser(473) in Akt/PKB, followed by the activation of Akt/PKB itself (Alessi et al., 1996; Alessi et al., 1997). The activated Akt/PKB is then known to regulate various cellular physiological responses, such as control of cell survival (Datta et al., 1997; del Peso et al., 1997), activation of glycogen synthesis (Shaw et al., 1997), promotion of glucose uptake (Kohn et al., 1996), cell transformation (Marte et al., 1997) as well as the induction of E2F transcriptional functions (Brennan et al., 1997). All of the Akt/PKB isoforms have the conserved threonine and serine residues, and the phosphorylation of their conserved residues is known to be an essential process for the full activation of Akt/PKB (Thomas et al., 2002). Various functional studies on Akt/PKB isoforms have reported that Akt1/PKBα is more likely to be involved in the regulation of cell growth, neonatal mortality and adipogenesis (Cho et al., 2001b; Garofalo et al., 2003; Yang et al., 2003), whereas Akt2/PKBβ is associated with the regulation of glucose metabolism (Cho et al., 2001a; Garofalo et al., 2003) and Akt3/PKBγ was shown to control the brain size in developing embryo (Easton et al., 2005). The diagram of

traditional PI3K-Akt/PKB signaling pathway is shown in Figure 4 (Kong and Yamori, 2010).

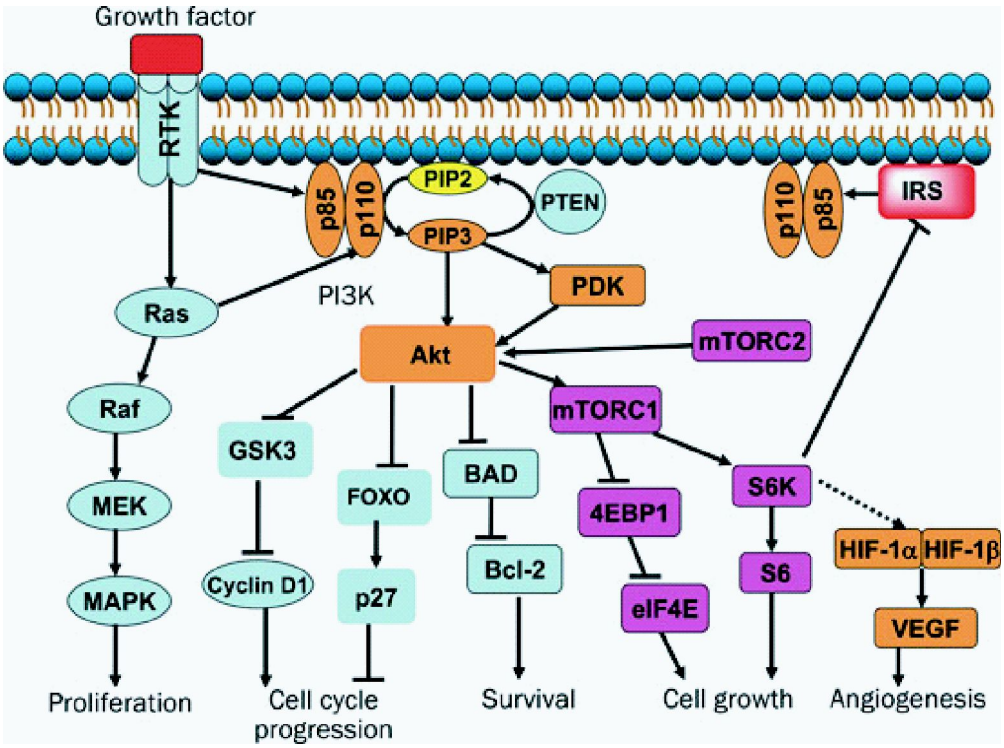
Previous studies from our laboratory demonstrated that 8-Cl-cAMP and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, an AMPK activator) induced the growth inhibition of cancer cells through the activation of AMPK and p38 MAPK (Han et al., 2009). In addition, other research group also reported that the growth inhibition of various cancer cells were promoted through the activation of AMPK and p38 MAPK (Lucchi et al., 2011). Accordingly, it was anticipated that the phosphorylation and activation of Akt/PKB would be decreased during 8-Cl-cAMP-induced cancer cell growth inhibition, because Akt/PKB was known to be actively involved in cell survival pathways by inhibiting apoptotic cell death (Datta et al., 1997; del Peso et al., 1997). Contrary to the above expectations, preliminary data obtained showed that the phosphorylation of Akt/PKB was increased by the treatment with 8-Cl-cAMP and AICAR in HeLa cells. Recent studies also indicated that various signal pathways were able to activate both the Akt/PKB and AMPK (Brazil and Hemmings, 2001; Horie et al., 2008). Based on this information, I decided to pursue the idea that activated Akt/PKB might induce the activation of AMPK and p38 MAPK during 8-Cl-cAMP- and AICAR-induced growth inhibition of cancer cells.

To find other signaling factor(s) working in and around the AMPK and p38 MAPK in 8-Cl-cAMP-induced cancer cell growth inhibition, I performed

Figure 4. The traditional signaling pathway of PI3K - Akt/PKB

The PI3K is activated through the interaction of extracellular signaling ligands with various receptor tyrosine kinases (RTKs), and this activated PI3K stimulates the activation of Akt/PKB through the activation of PDK1 and PDK2. The activated Akt/PKB is known to regulate the various cellular responses through the regulation of many different signaling factors (Kong and Yamori, 2010).

mTORC1/2: mTOR complex 1/2, IRS: insulin receptor substrate, BAD: Bcl2 associated death promoter, GSK3: glycogen synthesis kinase, FOXO: forkhead box O subclass, 4EBP1: 4E-binding protein 1, p70S6K: p70S6 kinase, HIF-1: hypoxia-inducible factor 1, VEGF: vascular endothelial growth factor, PTEN: phosphatase and tensin homolog


Growth factor
RTK
p85
p110
PIP2
PIP3
PTEN
PI3K
Ras
PDK
Akt
mTORC2
mTORC1
Raf
MEK
MAPK
GSK3
Cyclin D1
FOXO
p27
BAD
Bcl-2
4EBP1
eIF4E
S6K
S6
IRS
HIF-1α
HIF-1β
VEGF
Proliferation
Cell cycle progression
Survival
Cell growth
Angiogenesis

immunoprecipitation and mass spectrometry assay using anti-AMPK and anti-p38 antibodies. The SHC1 (Src homology 2 domain containing transforming protein 1) protein was selected as one of the binding partners of p38 MAPK by this assay, whole binding activities were influenced by the treatment of 8-Cl-cAMP (Table 1).

SHC1 protein was first identified in 1992 as an adaptor protein containing Src homology 2 (SH2) domains, which are conserved regions among a group of cytoplasmic signaling proteins (Pelicci et al., 1992; Ravichandran, 2001). The activities of signaling proteins containing SH2 domains were shown to be regulated by family of receptor tyrosine kinase and these proteins were known to control the activation of diverse signal transduction pathways (Koch et al., 1991; Ullrich and Schlessinger, 1990). SHC1 protein is known to be involved in the signaling pathways of numerous cell surface receptors such as growth factor receptors (Gelderloos et al., 1998; Pelicci et al., 1992; Pronk et al., 1994), antigen receptors (Ravichandran et al., 1993), cytokine receptors (Pratt et al., 1996; Velazquez et al., 2000), G-protein coupled receptors (Chen et al., 1996) and hormone receptors (Kousteni et al., 2001; Morte et al., 1998). Interestingly, many types of tumor cells exhibited hyper-phosphorylation of SHC1 proteins compared with their normal counterparts (Biscardi et al., 1998; Stevenson and Frackelton, 1998). Also, the over-expression of SHC1 construct and dominant negative form of SHC1 (R175Q) construct in MMTV-PyMY mouse model of breast cancer showed that the SHC1 proteins induced the tumor initiation, growth and angiogenesis (Ahn et al., 2013).

SHC1 protein is expressed as three isoforms (46, 52 and 66 kDa), which was shown to stem from alternative RNA splicing or alternative translational initiation (Migliaccio et al., 1997; Pelicci et al., 1992). Through extensive studies, p46/p52 SHC1 proteins were shown to be involved in the regulation of Ras activation (Luzi et al., 2000), MAPK activation (Gines et al., 2010), apoptosis of cancer cells (Murayama et al., 2004), cell proliferation (Benetti et al., 2004) and macrophage differentiation (Csar et al., 2001), whereas p66 SHC1 protein is associated with oxidative stress-induced apoptosis and life-span extension (Kleman et al., 2010; Luzi et al., 2000; Migliaccio et al., 1999), tumor progression (Du et al., 2013), tumor proliferation and metastasis (Ma et al., 2010) and PEITC-induced apoptosis (Xiao and Singh, 2010).

In preliminary studies, I was able to show that the elevated phosphorylation level of SHC1 proteins in HeLa cells was decreased during 8-Cl-cAMP-induced growth inhibition, whereas the total SHC1 protein level was not altered. Accordingly, I tried in this thesis to show the involvement of SHC1 proteins in the signaling pathways related to the inhibition of cancer cell proliferation caused by the treatment with 8-Cl-cAMP.

In addition to showing the involvement of Akt/PKB and SHC1 proteins in 8-Cl-cAMP-induced cancer cell growth inhibition, I also tried to position these two proteins in the signal transduction pathways with regard to the previously studied signaling molecules such as AMPK and p38 MAPK, which are summarized and

marked with ??? in Figure 3. Hence, the purpose(s) of this thesis study can be summarized as follows.

1. How do the Akt/PKB and SHC1 proteins be changed by the treatment of 8-Cl-cAMP?
2. Are the Akt/PKB and SHC1 involved in the activation of AMPK and p38 MAPK in 8-Cl-cAMP-induced cancer cell growth inhibition?
3. Can the changes in Akt/PKB and SHC1 proteins regulate the cancer cell growth?
4. What is the working order for the Akt/PKB and SHC1 proteins with regard to the AMPK and p38 MAPK proteins in this signaling pathway?

It was hoped through the studies in this thesis that I would be able to fill up some gaps related to the signaling pathways of 8-Cl-cAMP leading to the growth inhibition and/or apoptosis in cancer cells. I would be delighted if the outcome of this study would help to potentiate the use of 8-Cl-cAMP as a non-chemotherapeutic but as an anti-tumorigenic, physiological therapeutic agent in dealing with the cancer.

MATERIALS AND METHODS

Reagents and Antibodies

8-Cl-cAMP was purchased from Biolog (Bremen, Germany). AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) 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*, *Akt-2* and *SHC1* siRNA were produced by ST Pharm (Seoul, Korea). The nucleotide 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 sequence of the *SHC1* siRNA was as follows: sense - UGC CAA AGA CCC UGU GAA UdTdT; antisense - AUU CAC AGG

GUC UUU GGC AdTdT. The transfection reagent, LipofectamineTM 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, total-Akt1, total-Akt2, total-Akt3, total-AMPKα and phospho-SHC1 (Tyr239/240) 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 antibodies for c-myc, actin and total SHC1 were 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). HDF (Human Dermal Fibroblast) cells were cultured in DMEM medium (Hyclone, Logan, UT). The EMEM and RPMI1640 were supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin G, and 100 μg/ml

streptomycin. The DMEM was supplemented with 15% fetal bovine serum (Hyclone). Cultures were maintained in a humidified atmosphere of 5% CO2 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 hrs, 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 blotting

Cells were harvested by centrifugation and washed with PBS. The cell pellet was suspended in an extraction buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% NP40, 100 μM PMSF, 0.1 mM sodium pyrophosphate, 2.5 mM NaF, 1 μg/ml each of Na3VO4, benzamidine, aprotinin, antipain, and leupeptin) and incubated on ice for 30 min. After centrifugation at 14,000 x g for 15 min at 4°C, the supernatant was taken as cell extract. The extracts were separated on 10% SDS/PAGE and transferred onto a PVDF membrane. The protein-bound membrane was incubated with the appropriate antibodies followed by horse-radish peroxidase-conjugated anti-mouse or rabbit IgG anti-body (Bio-Rad, Hercules, CA). The relevant protein bands were then visualized using the ECLTM detection kit (Amersham).

Transfection of siRNA

One day before transfection, 3 x 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*, *SHC1* 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 hrs.

DNA fragmentation assay

Harvested cells were suspended in lysis buffer (5 mM Tris-Cl, pH 7.4; 20

mM EDTA; 0.5% Triton X-100), and incubated on ice for 30 min. After centrifugation at 14,000 x g for 30 min at 4°C, fragmented DNA in the supernatant was purified by phenol/chloroform extraction and precipitated with ethanol. DNA was then electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide.

Flow cytometry

Cells were fixed with ice-cold 70% ethanol for at least 3 hrs, and then stained with PI (50 μg/mL) containing RNase A (50 μg/mL) and Annexin V-FITC (50 μg/mL) at 37°C for 30 min. DNA content was analyzed by FACS-Calibur™ flow cytometer (BD Bioscience, San Jose, CA) using CellQuest™ programs (BD Bioscience).

Immunoprecipitation assay

Cultured cells were collected and washed with cold PBS and resuspended in extraction buffer (25 mM HEPES (pH 7.4), 50 mM NaCl, 1% Triton X-100, 10% glycerol, 0.1 mM PMSF, 0.1 mM pepstatin, 0.1 mM antipain, 0.2 mM leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 15 mM NaF and 1 mM Na3VO4). The suspension was then incubated on ice for 15 min. After lysates were clarified by centrifugation at 14,000 × g for 10 min, cleared protein extracts (2 mg) were incubated with appropriate antibodies overnight at 4˚C with continuous agitation. To

collect immune complexes, 50 µl of protein A Sepharose 4B (GE Healthcare Bio-Sciences) was added to the mixture and incubated for a further 2 hrs. The immunoprecipitates was washed three times with extraction buffer and then resuspended in 1x SDS-PAGE sample buffer. Followed by SDS-PAGE, bound proteins were analyzed by immunoblotting as well as mass spectrometry.

RESULTS

PART 1. Role of Akt2/protein kinase B β (PKBβ) in 8-Cl-cAMP-induced cancer cell growth inhibition

1. Treatment with 8-Cl-cAMP and AICAR induce the increased phosphorylation of Akt/PKB.

8-Cl-cAMP has been investigated as a potential anti-cancer drug, which induces the cancer cell-specific cellular growth inhibition and/or apoptosis in various cancer cell lines (Ciardiello et al., 1990; Kim et al., 2001). Also, since activation of Akt/PKB was well known to act as a cell survival signal in many cell types (Datta et al., 1997; del Peso et al., 1997), it was expected that the treatment with 8-Cl-cAMP and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, an AMPK activator) in cancer cells would decrease the level of phosphorylation of the Akt/PKB proteins and of their activation. Contrary to this expectation, preliminary results showed that the phosphorylation of Akt/PKB was increased by the treatment of 8-Cl-cAMP in HeLa cells, which leaded to further investigation.

First, I tested whether the phosphorylation level of Akt/PKB was changed during the course of 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 analysis was performed to measure the level of phosphorylation in Akt/PKB using antibodies against phospho-Akt1/2/3 and total Akt1/2/3 (Fig. 5A). All Akt/PKB isoforms were known to be phosphorylated at their conserved threonine(308) and serine(473) residues for full activation of the enzyme (Thomas et al., 2002). It was noted that the phosphorylation level of Akt/PKB was increased from 24-48 hrs after the treatment with 8-Cl-cAMP in a time-dependent manner. I also tested the effect of AICAR on the phosphorylation of Akt/PKB, because previous studies from the laboratory demonstrated that AICAR shared the same pathway as 8-Cl-cAMP in inhibiting cancer cell proliferation, i.e., through the activation of AMPK and p38 MAPK (Han et al., 2009). When HeLa cells were incubated with AICAR for 6 hrs, the phosphorylation of Akt/PKB was increased in a time-dependent manner (Fig. 5B). As previously reported, the phosphorylation of both the AMPK and p38 MAPK were increased upon treatments with 8-Cl-cAMP and AICAR (Figs. 5A, 5B).

Studies from a number of different research groups showed that the conversion of 8-Cl-cAMP inside the cell to 8-Cl-adenosine, one of the metabolites of 8-Cl-cAMP, was an essential process for the growth inhibitory effects in cancer cells (Ahn et al., 2005; Cho-Chung, 1991; Han et al., 2009; Lange-Carter et al., 1993). Accordingly, it was tested whether the conversion of 8-Cl-cAMP to its metabolites was essential for the phosphorylation of Akt/PKB. Treating HeLa cells with 8-Cl-Adenosine (10 μM), a metabolite of 8-Cl-cAMP, proved to enhance the

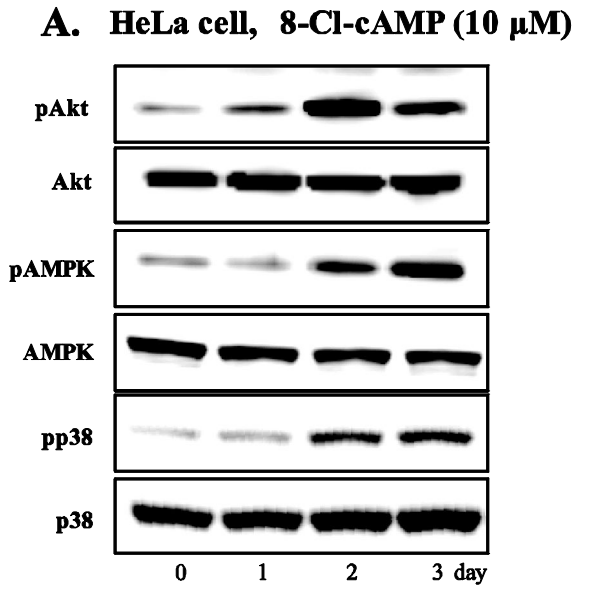
phosphorylation of Akt/PKB, AMPK as well as p38 MAPK just as 8-Cl-cAMP did (Fig. 6A). To determine if the phosphorylation of Akt/PKB by 8-Cl-cAMP was unique to HeLa cells, other cancer cell lines were also tested, such as SW480 (human colon cancer), DLD1 (human colon cancer), and MCF7 (human breast cancer). It was found that treatment with 8-Cl-cAMP increased the phosphorylation of Akt/PKB in all the cancer cell lines tested in a time dependent manner (Fig. 6B).

To confirm that the phosphorylation of Akt/PKB was indeed a direct 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, Figs. 7A & 7B) and ABT702 (an adenosine kinase inhibitor, Figs. 8A & 8B) for 1 hr before the treatment with 8-Cl-cAMP and AICAR and the phosphorylation pattern of respective molecules in a time course was monitored by Western blot analysis. As shown in Figures. 7 and 8, the increased phosphorylation of Akt/PKB, AMPK as well as p38 MAPK by AICAR and 8-Cl-cAMP were effectively attenuated by the pre-treatment with NBTI and ABT702. Furthermore, the phosphorylation of mTOR, a known downstream effector of Akt/PKB, was also decreased upon treatment with NBTI (Fig. 7B).

These data collectively indicate 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

Figure 5. Akt/PKB activity was increased after 8-Cl-cAMP and AICAR treatment.

A, B. HeLa cells were treated with 8-Cl-cAMP (10 μM) and AICAR (2 mM, an AMPK activator) 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.


A. HeLa cell, 8-Cl-cAMP (10 μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
0
1
2
3 day

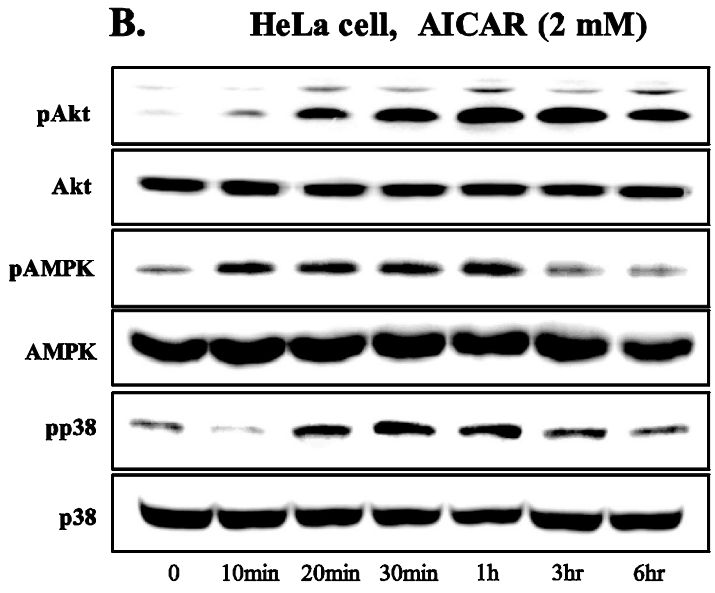

B. HeLa cell, AICAR (2 mM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
0
10min
20min
30min
1h
3hr
6hr

Figure 6. The treatment with 8-Cl-cAMP and 8-Cl-adenosine induced the phosphorylation of Akt/PKB in various cancer cell lines.

A. HeLa cell were treated with 8-Cl-Adenosine (10 μM) for the indicated times, and Western blot was performed as in Fig. 1A. ***B.*** SW480, DLD1 and MCF7 cells were treated with 8-Cl-cAMP (10 μM) for the indicated times, and Western blotting was performed using anti-phospho-Akt (pAkt) and anti-Akt (Akt) antibodies.

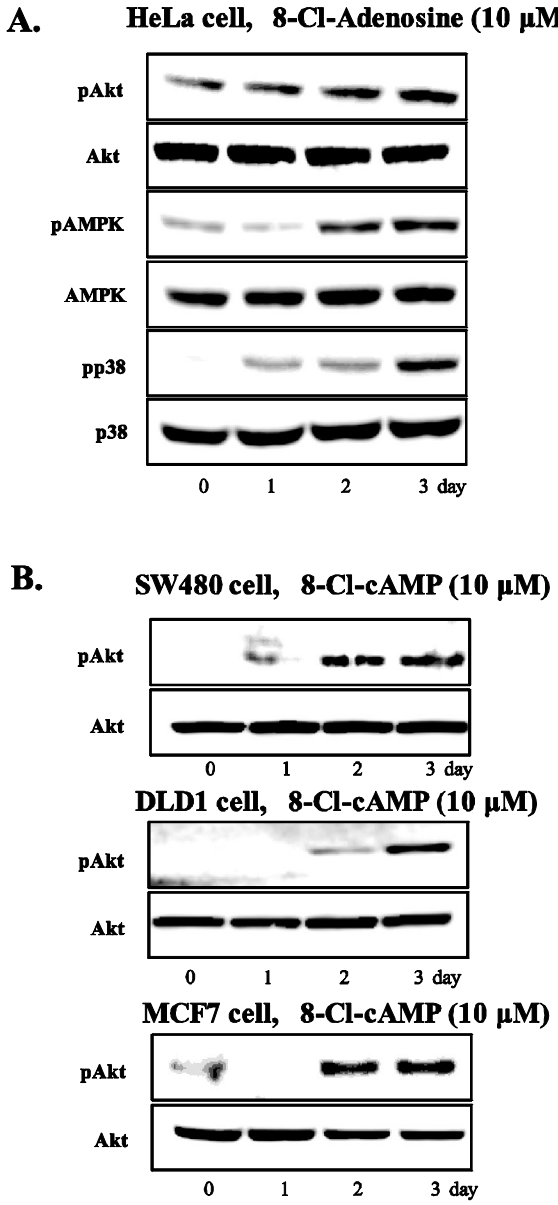
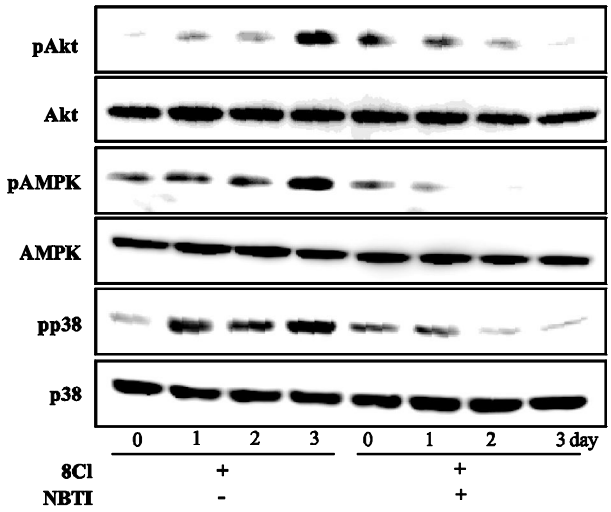

A.
HeLa cell, 8-Cl-Adenosine (10 μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
0 1 2 3 day
B.
SW480 cell, 8-Cl-cAMP (10 μM)
pAkt
Akt
0 1 2 3 day
DLD1 cell, 8-Cl-cAMP (10 μM)
pAkt
Akt
0 1 2 3 day
MCF7 cell, 8-Cl-cAMP (10 μM)
pAkt
Akt
0 1 2 3 day

Figure 7. 8-Cl-cAMP and AICAR treatment resulted in Akt/PKB phosphorylation.

A, B. HeLa cells were treated with 8-Cl-cAMP and AICAR for the indicated times in the presence or absence of NBTI (an adenosine transporter inhibitor, 100 μM). 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.

8Cl: 8-Cl-cAMP

A. HeLa cell, 8-Cl-cAMP (10 μM) ± NBTI (100 μM)



B. HeLa cell, AICAR (2 mM) ± NBTI (100 μM)

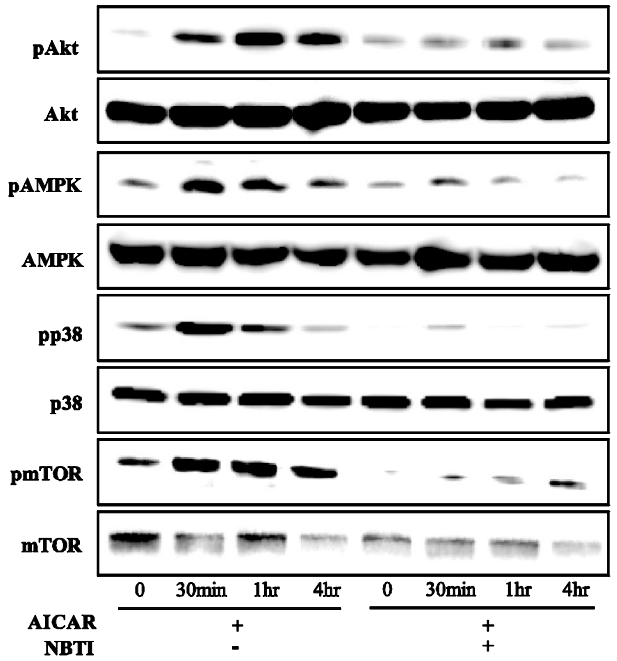
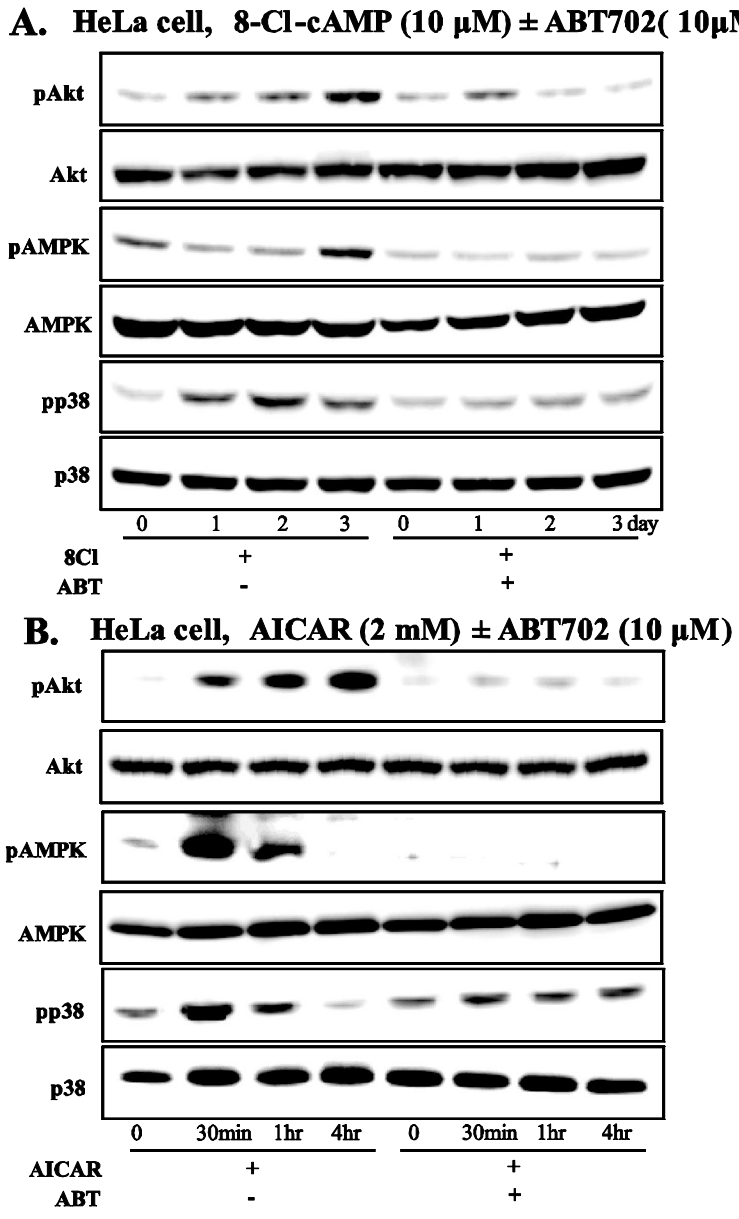


Figure 8. 8-Cl-cAMP and AICAR treatment resulted in Akt/PKB phosphorylation.

A, B. 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 μM). 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.

8Cl: 8-Cl-cAMP, ABT: ABT702


A. HeLa cell, 8-Cl-cAMP (10 μM) ± ABT702(10μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
0 1 2 3 0 1 2 3 day
8Cl + +
ABT - +
B. HeLa cell, AICAR (2 mM) ± ABT702 (10 μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
0 30min 1hr 4hr 0 30min 1hr 4hr
AICAR + +
ABT - +

transporter and then converted to their metabolites.

2. Anti-proliferative properties of 8-Cl-cAMP are confined to cancer cells.

A number of independent research groups have claimed that 8-Cl-cAMP induced growth inhibition and/or apoptosis in cancer cells, whereas it has almost no effect in non-transformed counterparts (Ahn et al., 2006; Ciardiello et al., 1990; Kim et al., 2001). For this thesis work, it seemed imperative to check once more whether the treatment of 8-Cl-cAMP is able to stimulate the phosphorylation of Akt/PKB, AMPK and p38 MAPK in non-transformed cells let alone the anti-proliferative activity. I employed HDF (Human Dermal Fibroblast) cells as non-transformed cell model and the effects of 8-Cl-cAMP in growth inhibition and cell death were monitored by cell counting, PI staining as well as the microscopic analysis.

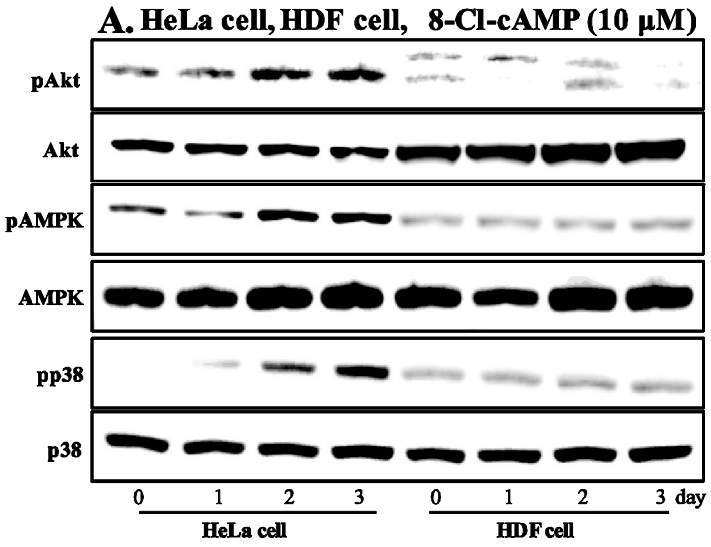
While 8-Cl-cAMP stimulated the phosphorylation of Akt/PKB, AMPK and p38 MAPK in HeLa cells, it did not elevate the phosphorylation level of the same signaling molecules in HDF cells (Fig. 9A). Also, while the proliferation of HeLa cells was significantly attenuated by 8-Cl-cAMP treatment, it did not show any inhibitory effect on the growth of HDF cells (Fig. 9B). In non-transformed HDF cells, the treatment of 8-Cl-cAMP did not seem to induce any remarkable cell death (PI-positive cells) either, as compared to the tumorigenic HeLa cells (Fig. 10).

These data clearly show that the treatment of 8-Cl-cAMP promotes the

Figure 9. The anti-proliferative activity of 8-Cl-cAMP is limited to cancer cells.

A. HeLa cells 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 cells and HDF cells were incubated with 8-Cl-cAMP (10 μM) for 3 days and cells were counted using a CoulterTM 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).

con: control, 8Cl: 8-Cl-cAMP


A. HeLa cell, HDF cell, 8-Cl-cAMP (10 μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
0 1 2 3 0 1 2 3 day
HeLa cell
HDF cell

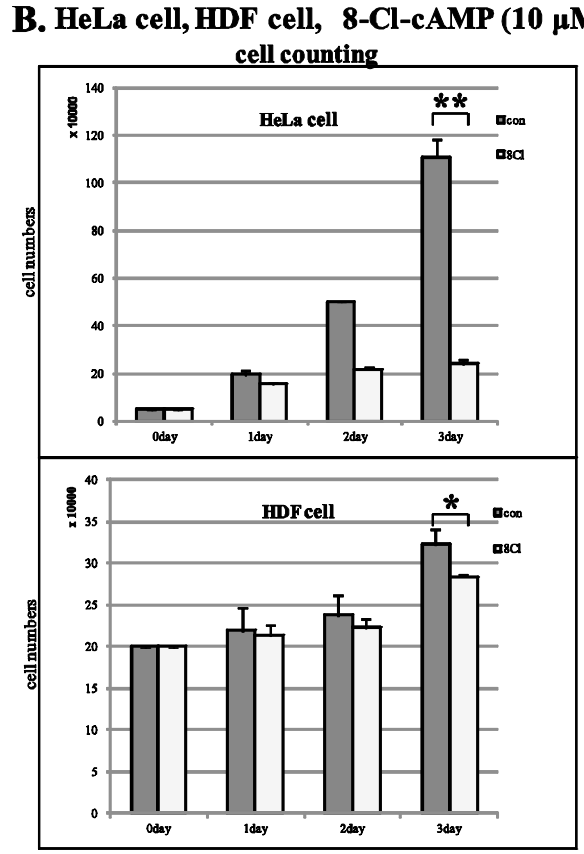
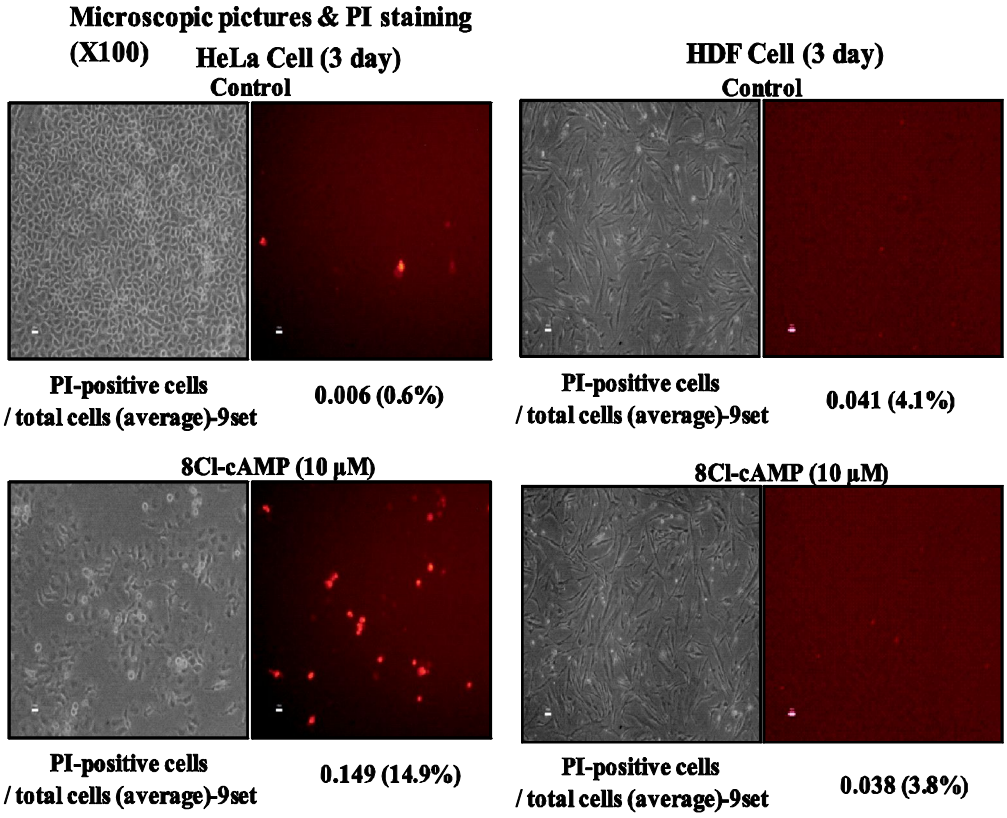

B. HeLa cell, HDF cell, 8-Cl-cAMP (10 μM)
cell counting
HeLa cell
**
con
8Cl
cell numbers
x 10000
0day
1day
2day
3day
HDF cell
*

Figure 10. Microscopic observation of 8-Cl-cAMP treated cells.

HeLa cells and HDF cells were incubated with 8-Cl-cAMP (10 μM) for 3 days, and then PI staining (8 μg/ml, 25 min) was performed to visualize the dead cells. Pictures were taken at 100X magnification.


Microscopic pictures & PI staining
(X100)
HeLa Cell (3 day)
Control
HDF Cell (3 day)
Control
PI-positive cells
/ total cells (average)-9set
0.006 (0.6%)
PI-positive cells
/ total cells (average)-9set
0.041 (4.1%)
8Cl-cAMP (10 μM)
8Cl-cAMP (10 μM)
PI-positive cells
/ total cells (average)-9set
0.149 (14.9%)
PI-positive cells
/ total cells (average)-9set
0.038 (3.8%)

phosphorylation and/or activation of Akt/PKB, AMPK and p38 MAPK only in transformed cancer cells and that this 8-Cl-cAMP induced anti-proliferative activity is limited to cancer cells, which are in line with the previous reports.

3. Akt/PKB seems to promote the activation of AMPK and p38 MAPK during 8-Cl-cAMP- or AICAR-induced growth inhibition.

Previous reports from the laboratory showed that the phosphorylation and activation of AMPK and p38 MAPK played a critical role during 8-Cl-cAMP-induced growth inhibition (Han et al., 2009). Accordingly, it was decided to see whether the phosphorylation of Akt/PKB is 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 hr of pre-treatment with Triciribine (TCN, an Akt1/2/3-specific inhibitor) (Figs. 11A and 11B, respectively). The AICAR-induced phosphorylation of Akt/PKB was reduced upon pre-treatment with TCN. Moreover, the phosphorylation of AMPK and p38 MAPK were decreased upon pre-treatment with TCN. The phosphorylation of mTOR, which represents the activation of Akt/PKB, was also suppressed by pre-treatment with TCN. Considering the results obtained with the co-treatment of the inhibitor TCN, it is safe to say that the phosphorylation and activation of Akt/PKB were effectively blocked by TCN, and accordingly the phosphorylation, which were induced by the treatment of both AICAR (Fig. 11A) and 8-Cl-cAMP (Fig. 11B), of AMPK, p38

MAPK as well as the mTOR, a known downstream effector of Akt/PKB, were effectively abolished in HeLa cells.

The involvement of PI3K, a well-known upstream activator of Akt/PKB, and mTOR, a known downstream effector of Akt/PKB, in the activation of Akt/PKB during the 8-Cl-cAMP induced growth inhibition was also investigated. Interestingly, while Akt-specific inhibitor, TCN, could repress the AICAR or 8-Cl-cAMP-induced phosphorylation of AMPK and p38 MAPK (Figs. 11A and 11B, respectively), the PI3K inhibitor LY294002 could not inhibit the phosphorylation of the respective signaling molecules, i.e., Akt/PKB, AMPK and p38 MAPK (Fig. 12A). Also, 8-Cl-cAMP-induced cancer cell growth inhibition was not attenuated by concomitant with LY294002 and Rapamycin, a known mTOR inhibitor (Figs. 12B and 12C, respectively). These results indicate that PI3K and mTOR are 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 activation of AMPK and p38 MAPK were dependent upon the activity of Akt/PKB during 8-Cl-cAMP- and AICAR-induced growth inhibition in HeLa cells.

To further confirm the involvement of the Akt/PKB in 8-Cl-cAMP-induced growth inhibition, siRNA approach was employed. I designed two different siRNAs targeted against *Akt/PKB*. One was targeted against *Akt1/PKBα* (*Akt-1* siRNA), and

Figure 11. Akt/PKB induced the phosphorylation of AMPK and p38 MAPK.

A. HeLa cells were incubated with TCN (an Akt1/2/3 specific inhibitor, 10 μM) for 1 hr prior to AICAR treatment (2 mM) for 4 hrs. Akt/PKB, AMPK and p38 MAPK activation was assessed by Western blot with phospho-specific antibodies as in Fig. 7B. The decreases in mTOR phosphorylation upon TCN treatment indicated that the activity of Akt/PKB was attenuated by TCN. ***B.*** HeLa cells were incubated with 8-Cl-cAMP (10 μM) for 1, 2 and 3 days with or without TCN (10 μM). Western blotting was performed as in Fig. 7B.

8Cl: 8-Cl-cAMP

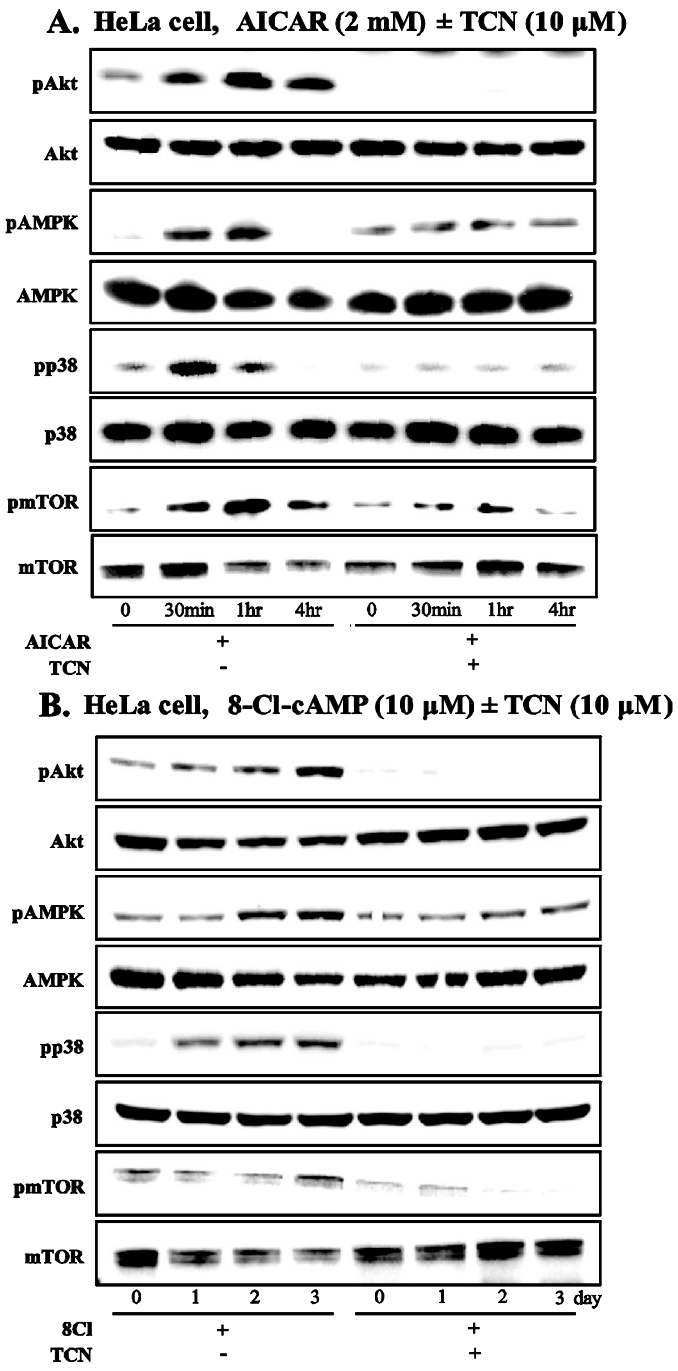
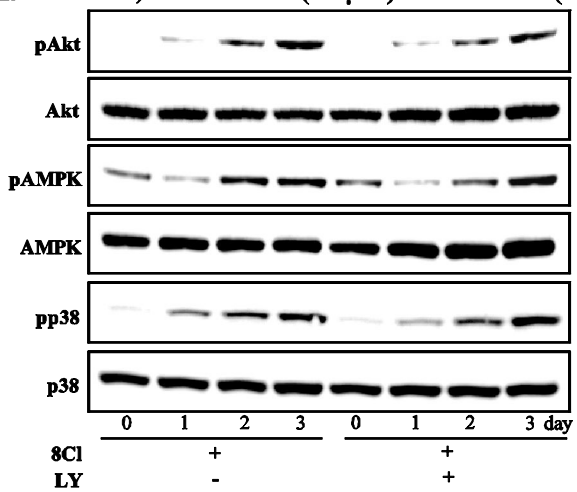

A. HeLa cell, AICAR (2 mM) ± TCN (10 μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
pmTOR
mTOR
0 30min 1hr 4hr 0 30min 1hr 4hr
AICAR + +
TCN - +
B. HeLa cell, 8-Cl-cAMP (10 μM) ± TCN (10 μM)
pAkt
Akt
pAMPK
AMPK
pp38
p38
pmTOR
mTOR
0 1 2 3 0 1 2 3 day
8Cl + +
TCN - +

Figure 12. LY294002 and Rapamycin did not attenuate the activation of 8-Cl-cAMP-induced cancer cell growth inhibition.

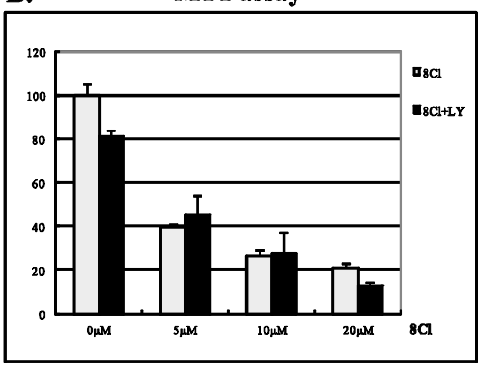
A. HeLa cells were pre-treated with LY294002 (a PI3K specific inhibitor, 10 μM) for 1 hr prior to 8-Cl-cAMP (10 μM) treatments. Western blotting was performed as in Fig. 5. ***B.*** HeLa cells was incubated with 8-Cl-cAMP (5, 10, 20 μM) for 3 days with or without LY294002 (5, 10 μM), and the viability of cells was measured using MTT assay analysis. Bars denote cell viability normalized to the control group (mean±SD, n=9). ***C.*** HeLa cells was incubated with 8-Cl-cAMP (10 μM) for 3 days with or without Rapamycin (1 μM), and the cells were counted using a Coulter^{TM} counter. Bars denote cell number (mean±SD, n=9).

8Cl: 8-Cl-cAMP, LY: LY294002, Rapa: Rapamycin

A. HeLa cell, 8-Cl-cAMP (10 μM) ± LY294002 (10 μM)

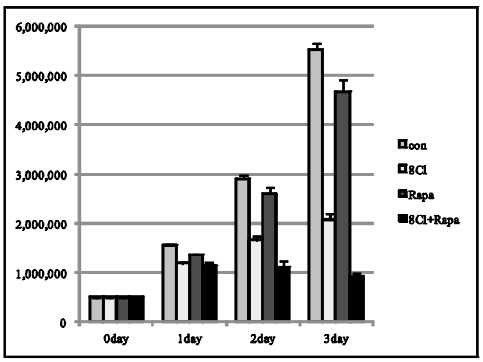


B. MTT assay



HeLa cell, 8-Cl-cAMP (10μ M) + LY294002 (5 μM) 3day

C. Cell counting



HeLa cell, 8-Cl-cAMP (10 μM) + Rapamycin (1 μM) 3day

the other was targeted against *Akt2/PKBβ* (*Akt-2* siRNA) (Fig. 13A). Western blot analysis showed that the expression level of Akt/PKB was significantly reduced 24 hrs after transfection with the *Akt-1* and *Akt-2* siRNAs (Fig. 13B), whereas the amount of both the AMPK and p38 MAPK protein were not fluctuated. By employing the Akt-1, Akt-2 and Akt-3 specific antibodies, I was able to show that *Akt-1* siRNA lowered the level of only Akt-1 protein, whereas *Akt-2* siRNA lowered only Akt-2 protein level (Fig. 14). It was also tested whether the transfection with *Akt-1* and *Akt2* siRNAs could affect the phosphorylation of AMPK and p38 MAPK upon treatment with 8-Cl-cAMP. As a control, it was found that a *GFP*-targeted siRNA did not block the phosphorylation of AMPK and p38 MAPK when exposed to 8-Cl-cAMP (Fig. 14). However, when HeLa cells were treated with *Akt-2* siRNA, the phosphorylation level of AMPK and p38 MAPK were markedly diminished, while transfection with *Akt-1* siRNA did not influence the phosphorylation pattern of AMPK, p38 MAPK, as significantly as *Akt-2* siRNA (Fig. 14).

These results demonstrated that only Akt2/PKBβ among the three Akt/PKB isoforms appeared to be more actively involved in the phosphorylation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells induced by 8-Cl-cAMP treatment.

Figure 13. The effect of siRNA targeted against *Akt2/PKBβ* on the expression of Akt2/PKBβ protein.

***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. Western blot analysis was performed with appropriate antibodies.

A. **Sequences of siRNAs targeted to *Akt/PKB***

→ ***Akt-1*** **: targeted siRNA of *Akt1/ PKBα***

Akt-1 **sense : GGA CAG AGG AGC AAG GUU UdTdT**

Akt-1 **antisense : AAA CCU UGC UCC UCU GUC CdTdT**

→ ***Akt-2*** **: targeted siRNA of *Akt2/ PKBβ***

Akt-2 **sense : CGA CUG AGG AGA UGG AAG UdTdT**

Akt-2 **antisense : ACU UCC AUC UCC UCA GUC GdTdT**

B. **HeLa cell, siRNA (*Akt-2*, *Akt-1*) (60 nM) transfection**

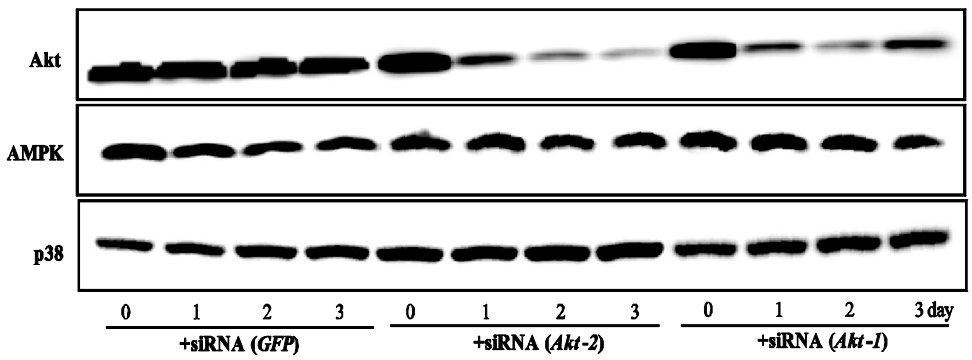
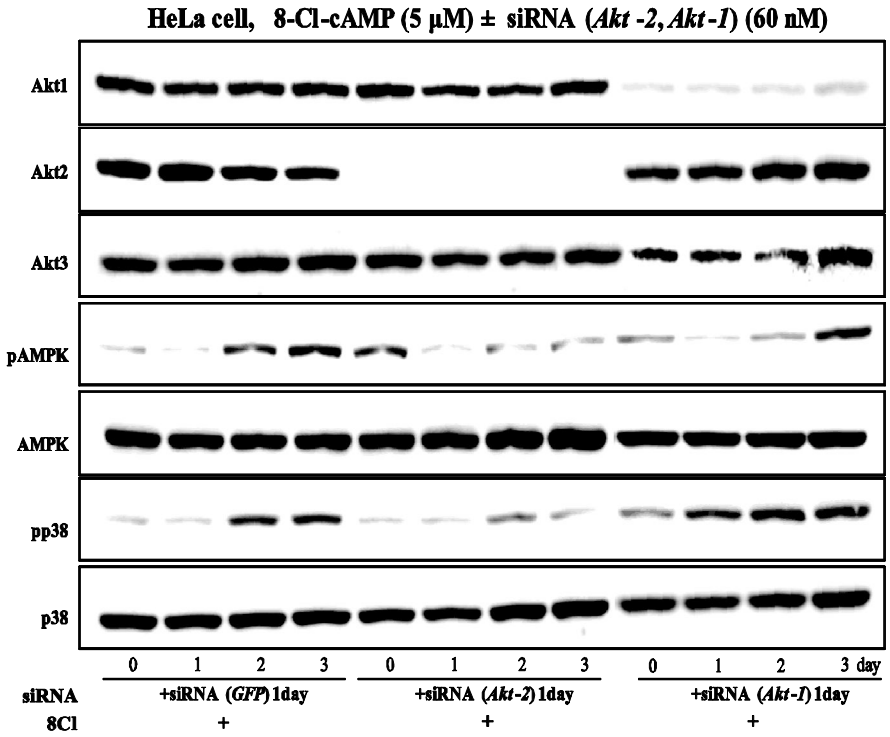


Figure 14. siRNA targeted against *Akt2/PKBβ* attenuated the activation of AMPK and p38 MAPK upon treatment with 8-Cl-cAMP.

HeLa cells were treated with 8-Cl-cAMP (5 μM) for the indicated times 24 hrs after transfection with *GFP*-targeted siRNA (60 nM), *Akt2/PKBβ*-targeted siRNA (60 nM) and *Akt1/PKBα*-targeted siRNA (60 nM). 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.

8Cl: 8-Cl-cAMP


HeLa cell, 8-Cl-cAMP (5 μM) ± siRNA (Akt -2, Akt -1) (60 nM)
Akt1
Akt2
Akt3
pAMPK
AMPK
pp38
p38
0 1 2 3 0 1 2 3 0 1 2 3 day
siRNA
+siRNA (GFP) 1day
+siRNA (Akt-2) 1day
+siRNA (Akt-1) 1day
8Cl
+
+
+

4. The Akt/PKB specific inhibitor, TCN, blocked the growth inhibition and cell death mediated by 8-Cl-cAMP.

To further confirm the direct involvement of Akt/PKB in growth inhibition and cell death mediated by 8-Cl-cAMP, several cancer cell lines were employed in MTT assays as well as growth analyses. First, to determine whether Akt/PKB inhibitor could reverse the growth inhibitory effect of 8-Cl-cAMP, TCN was co-treated with 8-Cl-cAMP in HeLa cells. The 8-Cl-cAMP-induced growth inhibition was effectively attenuated by co-treatment with TCN as determined by MTT assay (Fig. 15). Also, cell growth analysis with a number of cancer cell lines by cell counting analysis (Fig. 16) revealed similar reversal of growth inhibition caused by 8-Cl-cAMP treatment as in MTT assay.

The final approach to confirm the involvement of Akt/PKB in 8-Cl-cAMP-mediated cancer cell growth inhibition was to look for the apoptotic signature. As shown in Figure 17, the average number of PI-positive (dead) cells mediated by 8-Cl-cAMP treatment was decreased upon co-treatment with TCN. Finally, Annexin-V-FITC FACS analysis and DNA fragmentation assay were employed to determine whether the PI-positive dead cells (in Fig. 17) are of the apoptotic or necrotic nature (Figs. 18A and 18B, respectively). Annexin-V-FITC FACS analysis as well as DNA fragmentation assay point to the direction that apoptosis is not the major cause of cell death during 8-Cl-cAMP-induced growth inhibition at least in HeLa cells (Figs. 18A, 18B).

The data obtained so far through various approaches indicate that the growth inhibition and cell death caused by 8-Cl-cAMP treatment are indeed mediated through the activation of Akt/PKB and that the nature of cell death is not the apoptotic one.

5. Akt2/PKBβ plays a role as upstream factor of AMPK and p38 MAPK during 8-Cl-cAMP-induced growth inhibition.

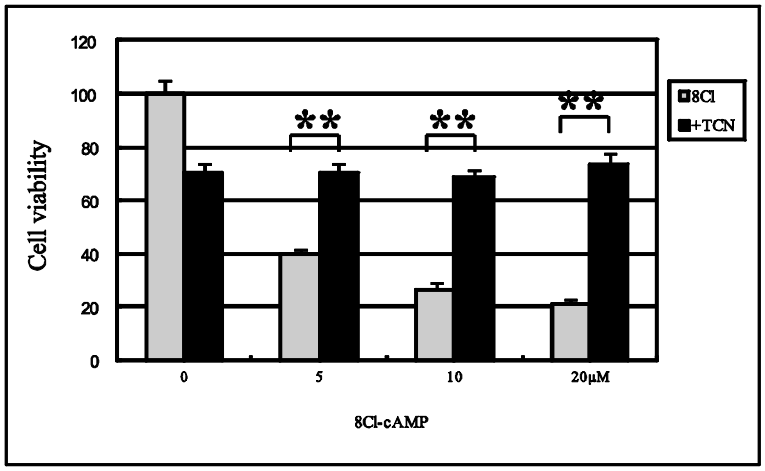
Thus far, it was shown that Akt2/PKBβ is mainly responsible for the phosphorylation and activation of AMPK and p38 MAPK during 8-Cl-cAMP-mediated growth inhibition. To clarify the working order of these signaling molecules, I employed some chemical inhibitors as well as mutant construct. The chemical inhibitor selective for AMPK, Compound C, inhibited the phosphorylation of both p38 MAPK and MAPKAPK2 (a downstream effector of p38 MAPK), whereas the phosphorylation of Akt/PKB was not significantly decreased by the treatment with Compound C during the 8-Cl-cAMP-induced growth inhibition in HeLa cells (Fig. 19A). The phospho-MAPKAPK2 specific antibody was used for measuring kinase activity of p38 MAPK. Then, to look at more closely the role of AMPK in this growth inhibition process, I used AMPK-DN construct (a dominant negative kinase, kinase dead mutant from of AMPK) and investigate its effect in HeLa cells. In this construct, the lysine residue for ATP binding and hydrolysis was changed to arginine by *in vitro* mutagenesis, resulting in the dominant negative

Figure 15. The Akt-specific inhibitor, TCN, blocked 8-Cl-cAMP-induced growth inhibition.

HeLa cells were incubated with 8-Cl-cAMP (0, 5, 10, 20 μM) for 3 days with or without TCN (10 μM), 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).

8Cl: 8-Cl-cAMP

MTT Assay



HeLa cell, 8-Cl-cAMP + TCN (10 μM)

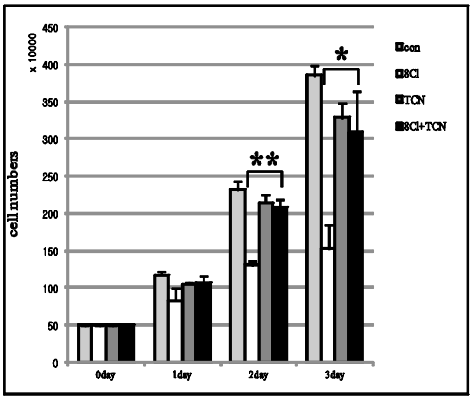
Figure 16. The Akt-specific inhibitor, TCN, blocked 8-Cl-cAMP-induced growth inhibition.

A, B, C. HeLa cells, DLD1 cells and SW480 cells were incubated with 8-Cl-cAMP (10 μM) for 3 days with or without TCN (10 μM), and cells were counted using a Coulter^{TM} 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).

8Cl: 8-Cl-cAMP

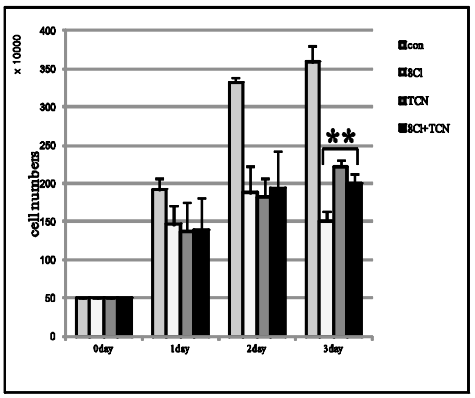
Cell counting

A.



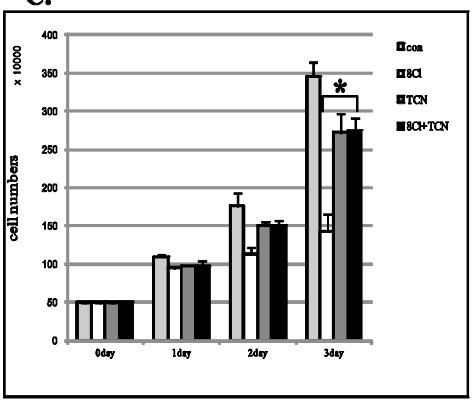
HeLa cell, 8-Cl-cAMP (10 μM) + TCN (10 μM)

B.



DLD1 cell, 8-Cl-cAMP (10 μM) + TCN (10 μM)

C.



SW480 cell, 8-Cl-cAMP (10 μM) + TCN (10 μM)

Figure 17. TCN blocked cell death mediated by 8-Cl-cAMP treatments.

The Akt-specific inhibitor reversed the 8-Cl-cAMP-induced growth inhibition and cell death. HeLa cells were incubated with 8-Cl-cAMP (10 μM) for 3 days with or without TCN (10 μM), and then PI staining (8 μg/ml, 25 min) was performed to visualize dead cells. Pictures were taken at 100X magnification.

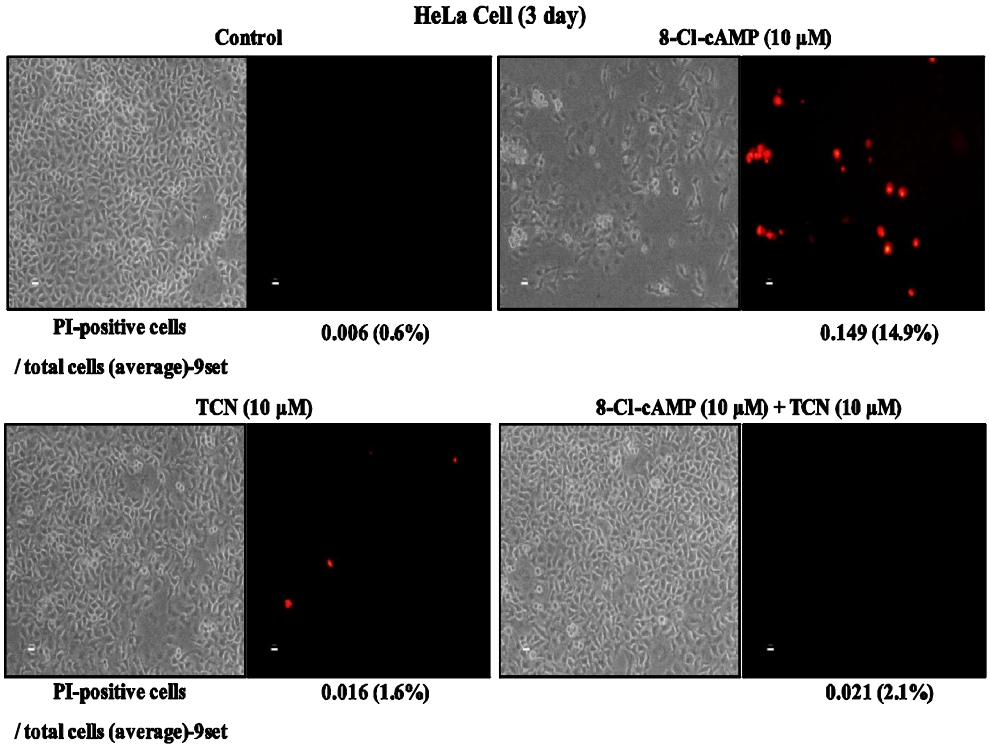
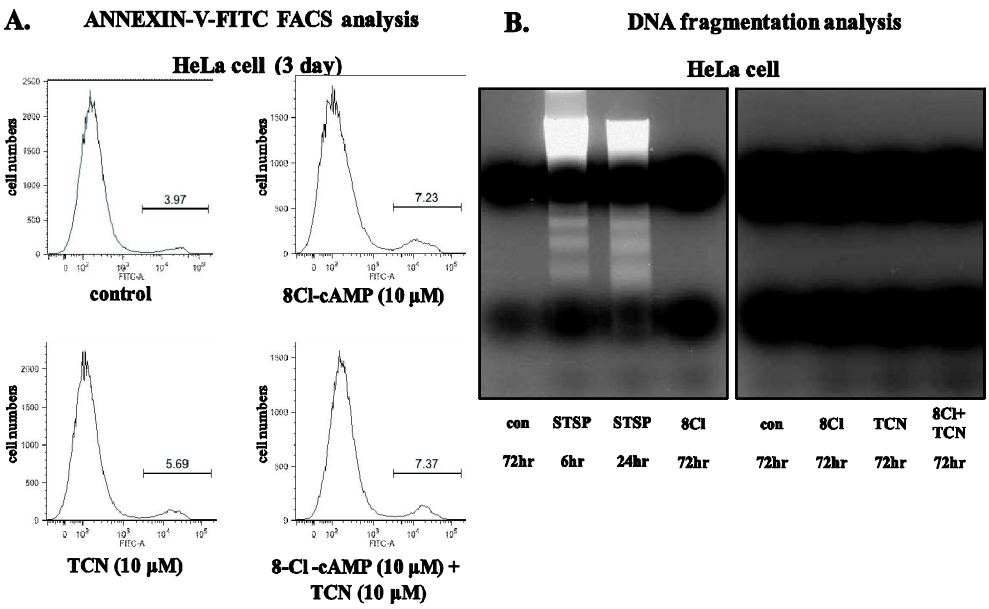

HeLa Cell (3 day)
Control
8-Cl-cAMP (10 μM)
PI-positive cells
/ total cells (average)-9set
0.006 (0.6%)
0.149 (14.9%)
TCN (10 μM)
8-Cl-cAMP (10 μM) + TCN (10 μM)
PI-positive cells
/ total cells (average)-9set
0.016 (1.6%)
0.021 (2.1%)

Figure 18. The cell death mediated by 8-Cl-cAMP treatments is not apoptosis.

A, B. HeLa cells were incubated with TCN (10 μM) for 1 hr prior to 8-Cl-cAMP treatment (10 μM) 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) according to the protocol of manufacturer. The positive control was the groups of STSP (Staurosporine) treatment (1 μM) for 6 hrs and 24 hrs in DNA fragmentation analysis.

con: control, 8Cl: 8-Cl-cAMP, STSP: Staurosporine


A.
ANNEXIN-V-FITC FACS analysis
HeLa cell (3 day)
cell numbers
FITC-A
3.97
control
7.23
8Cl-cAMP (10 μM)
5.69
TCN (10 μM)
7.37
8-Cl -cAMP (10 μM) +
TCN (10 μM)
B.
DNA fragmentation analysis
HeLa cell
con
STSP
STSP
8Cl
con
8Cl
TCN
8Cl+
TCN
72hr
6hr
24hr
72hr
72hr
72hr
72hr
72hr

phenotype of AMPK (Mu et al., 2001). Whereas the phosphorylation and/or activation of Akt/PKB was not meaningfully affected when AMPK-DN construct was over-expressed in 8-Cl-cAMP treated HeLa cells, the phosphorylation of both the p38 MAPK as well as its downstream effector, MAPKAPK2, were significantly diminished (Fig. 19B). In addition, the phosphorylation of MAPKAPK2 was blocked by SB203580, a p38 MAPK-specific inhibitor, whereas the phosphorylation of Akt/PKB and AMPK were not affected in 8-Cl-cAMP-treated cells (Fig. 19C).

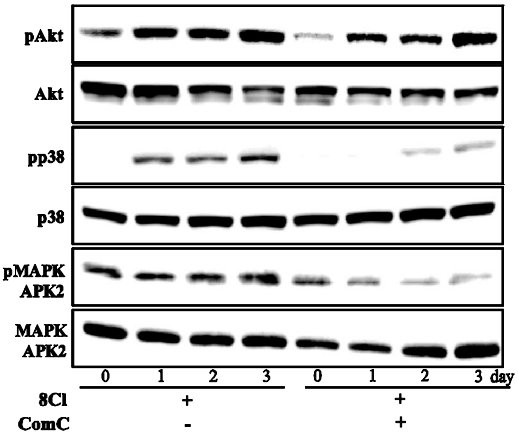
These results collectively place the working order for the three signaling molecules in this study, i.e., Akt/PKB plays most upstream role, AMPK in the middle and p38 MAPK works as downstream factor during 8-Cl-cAMP-induced growth inhibition and cell death in HeLa cells.

Figure 19. Akt/PKB acted upstream of AMPK and p38 MAPK during 8-Cl-cAMP-induced growth inhibition.

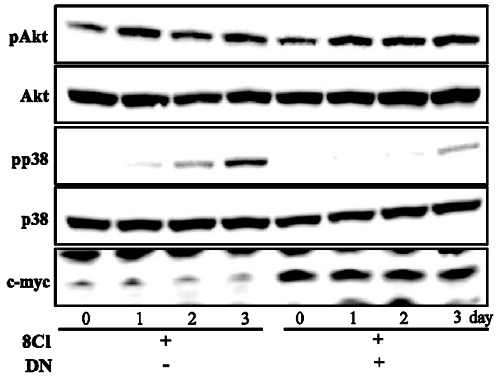
A. HeLa cells were pre-treated with Compound C (an AMPK specific inhibitor, 1 μM) for 1 hr prior to 8-Cl-cAMP (10 μM) for 3 days. Western blotting was performed with anti-phospho-Akt (pAkt), anti-Akt (Akt), anti-phospho-p38 MAPK (pp38), anti-p38 (p38), anti-phospho-MAPKAPK2 (pMAPKAPK2 or pMK2) and anti-MAPKAPK2 (MAPKAPK2 or MK2) antibodies. ***B.*** The expression of KD-AMPK-transfected stable cells (AMPK-DN) was confirmed by Western blotting using a c-myc antibody. HeLa cells and KD-AMPK-transfected cells were treated with 8-Cl-cAMP (10 μM) 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) and anti-p38 (p38) antibodies. ***C.*** HeLa cells were pre-treated with SB203580 (a p38 MAPK specific inhibitor, 1 μM) for 1 hr before treating with 8-Cl-cAMP (10 μM) for 3 days. Western blotting was performed as in A.

8Cl: 8-Cl-cAMP, ComC: Compound C, DN: AMPK dominant negative, SB: SB203580

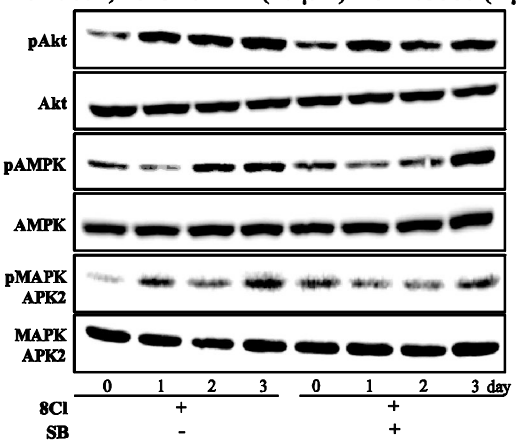
A. HeLa cell, 8-Cl-cAMP (10 μM) ± CompoundC (1 μM)



B. HeLa cell, 8-Cl-cAMP (10 μM) ± AMPK-DN



C. HeLa cell, 8-Cl-cAMP (10 μM) ± SB203580 (1 μM)



PART 2. Role of SHC1 protein in 8-Cl-cAMP-mediated cancer cell growth inhibition

1. The phosphorylation of SHC1 protein in cancer cells was decreased when treated with 8-Cl-cAMP.

In search of other signaling molecule(s) involving AMPK and p38 in 8-Cl-cAMP-induced cancer cell growth inhibition, I performed immunoprecipitation assay using anti-AMPK and anti-p38 antibodies. HeLa cells were incubated with or without 8-Cl-cAMP (10 μM) for 3 days and immunoprecipitation and mass spectroscopic analysis was performed to determine which protein(s) interacted with AMPK or p38 MAPK in 8-Cl-cAMP-induced cancer cell growth inhibition. Some candidate proteins were identified as the interaction partners of AMPK and p38 MAPK by this assay (Table 1). Among them, the SHC1 protein was selected for further study since its binding activity was significantly influenced by the treatment of 8-Cl-cAMP.

It has long been known that the Src homology domain 2 containing transforming protein 1 (SHC1) proteins are hyper-phosphorylated in various types of tumor cells compared to non-transformed counterparts (Biscardi et al., 1998; Stevenson and Frackelton, 1998). Also, there have been numerous reports describing

Table 1. The mass spectrometric analysis of immunoprecipitates using antibodies against AMPK and p38 MAPK

HeLa cells were incubated with or without 8-Cl-cAMP (10 μM) for 3 days, and immunoprecipitation assay was performed to determine which protein(s) interacted with AMPK or p38 MAPK during 8-Cl-cAMP-induced cancer cell growth inhibition. The protein bands of interest were then analyzed by mass spectrometric analysis to determine the identity of each bound proteins.

Mass Spectrometric Analysis	AMPK IP	p38 MAPK IP
Analyzed proteins	Prolyl 4-hydroxylase alpha polypeptide II (P4HA2) Brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2, IRSP53) B cell CLL/lymphoma 9 (BCL9) D4, zinc and double PHD finger, family 3 (DPF3) Ankyrin-3 (ANK3) Retinoblastoma binding protein 8 (RBBP8, CTIP) Protein tyrosine phosphatase, receptor type M (PTPRM) PDZ and LIM domain 2 (Mystique, PDLIM2) Cellular repressor of E1A-stimulated gene 1 (CREG1) Retinoblastoma binding protein 1 like 1 (RBBP1L1, ARID4B) Minichromosome maintenance complex component 7 (MCM7) Cyclin D-binding Myb-like transcription factor 1 isoform b (DMTF1, DMP1) FBJ murine osteosarcoma viral oncogene homolog B (FosB, AP-1)	**SHC (Src homology 2 domain containing) transforming protein 1 (SHC1)** Cooper-transporting ATPase2 isoform b (ATP7B) MYST1 histone acetyltransferase 2 (MYST2, HBO1) Mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2) Pleckstrin homology-like domain, family A, member 1 (PHLDA1, TDAG51) Brain expressed, x-linked 1 (BEX1) WNK lysine deficient protein kinase 2 (WNK2) GLI-Krupple related protein YY1 transcription factor (YY1, YIN-YANG1) SIN3 homolog A, transcription regulator (SIN3A) Death- associated protein kinase 1 (DAPK1) Ribosomal protein S6 kinase alpha-6 (RPS6KA6, p90RSK6, RSK4) THAP domain containing 11 (THAP11, RONIN) ATPase family, AAA domain containing 3B (ATAD3B) Angiopoietin 1 (ANGPT1, ANG1)

the physiological relevance of each isoforms of SHC1 in various cell lines. The p46 and p52 SHC1 isoforms have been known to regulate the MAPK activation (Gines et al., 2010), apoptosis of cancer cells (Murayama et al., 2004) and cell proliferation (Benetti et al., 2004), whereas p66 SHC1 protein was shown to be involved in the oxidative stress-induced apoptosis and life-span extension (Kleman et al., 2010; Luzi et al., 2000; Migliaccio et al., 1999) as well as tumor progression and metastasis (Du et al., 2013; Ma et al., 2010). With these characterized properties of SHC1 proteins, it was expected that the phosphorylation and/or activation of SHC1 proteins in cancer cells would be decreased upon treatment with 8-Cl-cAMP, because activated SHC1 was known to stimulate the cell proliferation and metastasis in many cancer cells. Preliminary results regarding this SHC1 phosphorylation showed that phosphorylation of SHC1 was decreased by the treatment with 8-Cl-cAMP in HeLa cells and MCF7 cells.

I decided to take a more closely look at the phosphorylation aspect of SHC1 protein during the 8-Cl-cAMP-mediated growth inhibition in HeLa cells and MCF7 cells. It was shown by careful and elegant analysis that three different tyrosine residues, Tyr(239), Tyr(240) and Tyr(317), on SH2 and PTB domains of SHC1 are phosphorylated by activated receptors for its full activation in response to extracellular signals (Gotoh et al., 1996; Salcini et al., 1994; van der Geer et al., 1996). HeLa and MCF7 cells (Figs. 20A and 20B, respectively) were incubated with 8-Cl-cAMP (10 μM) for 3 days, and Western blotting was performed to measure the

phosphorylation of SHC1 using antibodies against phospho-SHC1 and total SHC1. While the SHC1 phosphorylation was considerably decreased from 24-48 hrs after the treatment with 8-Cl-cAMP in a time-dependent manner, the phosphorylation of AMPK and p38 MAPK were increased in both HeLa cells and MCF7 cells (Fig. 20).

In addition, I tested the effect of metabolites of 8-Cl-cAMP treatment toward the phosphorylation of SHC1, because the conversion of 8-Cl-cAMP to its metabolites was shown to be an essential process for its growth inhibitory activity (Ahn et al., 2004; Ahn et al., 2005; Han et al., 2009). The treatment with 8-Cl-Adenosine (5 μM) and 8-Cl-ATP (5 μM) (Figs. 21A and 21B, respectively), both of which are the metabolites of 8-Cl-cAMP, also decreased the level of SHC1 phosphorylation. At the same time, both reagents promoted the phosphorylation of AMPK and p38 MAPK, just as 8-Cl-cAMP did in the previous experiments.

To confirm that the de-phosphorylation of SHC1 was actually a result of 8-Cl-cAMP treatment, HeLa cells were pre-treated with ABT702 (an adenosine kinase inhibitor) for 1 hr before treating with 8-Cl-cAMP. The de-phosphorylation of SHC1 and the phosphorylation of AMPK and p38 MAPK by 8-Cl-cAMP treatment were effectively blocked by concomitant treatment with ABT702 (Fig. 22), indicating that the de-phosphorylation of SHC1 protein was indeed caused by 8-Cl-cAMP treatment.

These data showed that treatment with 8-Cl-cAMP induced the down-regulation of the phosphorylation and/or activation of SHC1 in both the HeLa cells and MCF7 cells. Furthermore, the conversion of 8-Cl-cAMP to its metabolites was

Figure 20. SHC1 phosphorylation was decreased after 8-Cl-cAMP treatment.

A, B. HeLa cells and MCF7 cells were treated with 8-Cl-cAMP (10 μM) for the indicated times, and Western blotting was carried out using anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1), anti-phospho-AMPK (pAMPK), anti-AMPK (total AMPK), anti-phospho-p38 MAPK (pp38), anti-p38 MAPK (total p38) and anti-Actin (Actin) antibodies.

8Cl: 8-Cl-cAMP

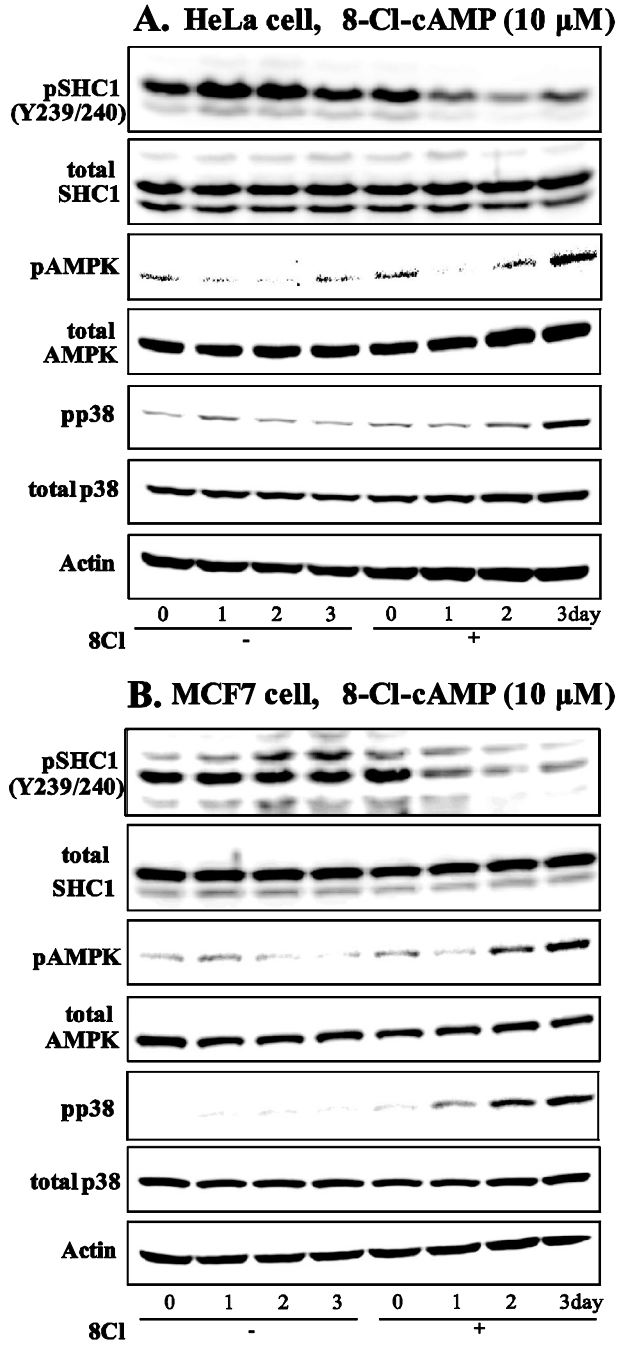

A. HeLa cell, 8-Cl-cAMP (10 μM)
pSHC1 (Y239/240)
total SHC1
pAMPK
total AMPK
pp38
total p38
Actin
0 1 2 3 0 1 2 3day
8Cl - +
B. MCF7 cell, 8-Cl-cAMP (10 μM)
pSHC1 (Y239/240)
total SHC1
pAMPK
total AMPK
pp38
total p38
Actin
0 1 2 3 0 1 2 3day
8Cl - +

Figure 21. The metabolites of 8-Cl-cAMP induced the decrease of SHC1 phosphorylation.

A, B. HeLa cells were treated with 8-Cl-adenosine (5 μM) and 8-Cl-ATP (5 μM) for the indicated times, and Western blotting was carried out using anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1), anti-phospho-AMPK (pAMPK), anti-AMPK (total AMPK), anti-phospho-p38 MAPK (pp38) and anti-p38 MAPK (total p38) antibodies.

8Cl-Ado: 8-Cl-adenosine, 8Cl-ATP: 8-Cl-ATP

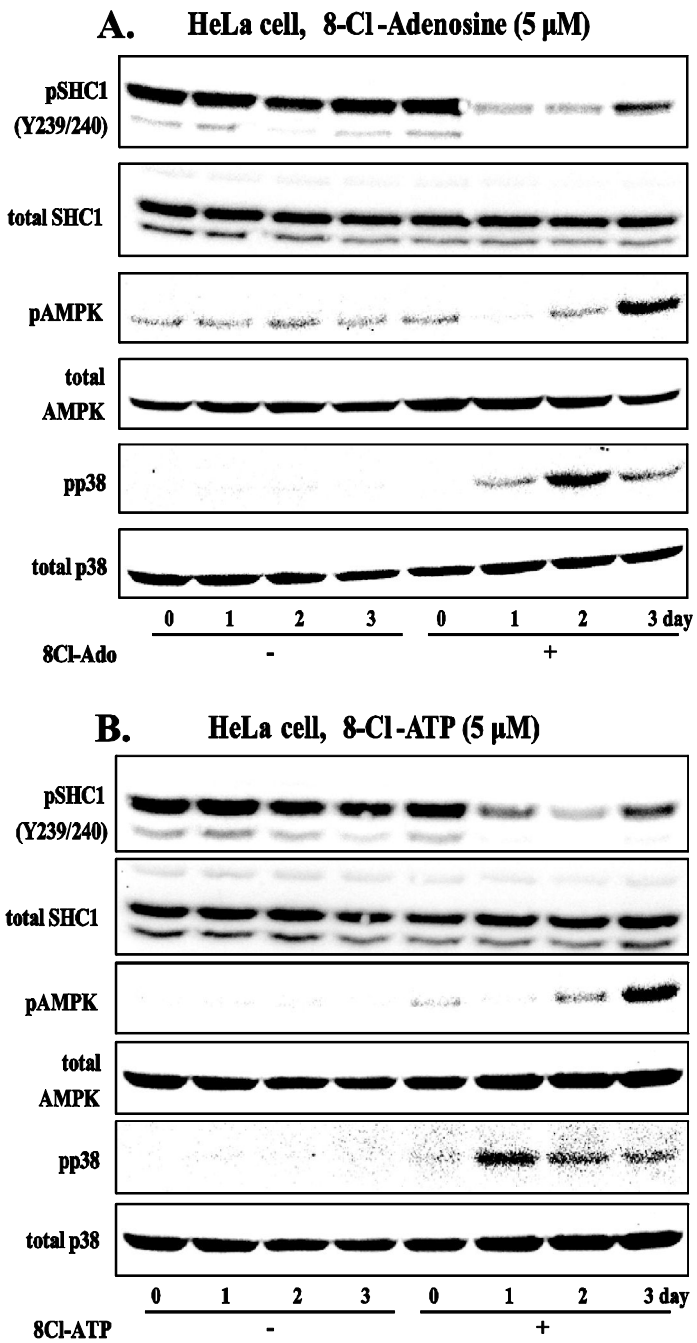
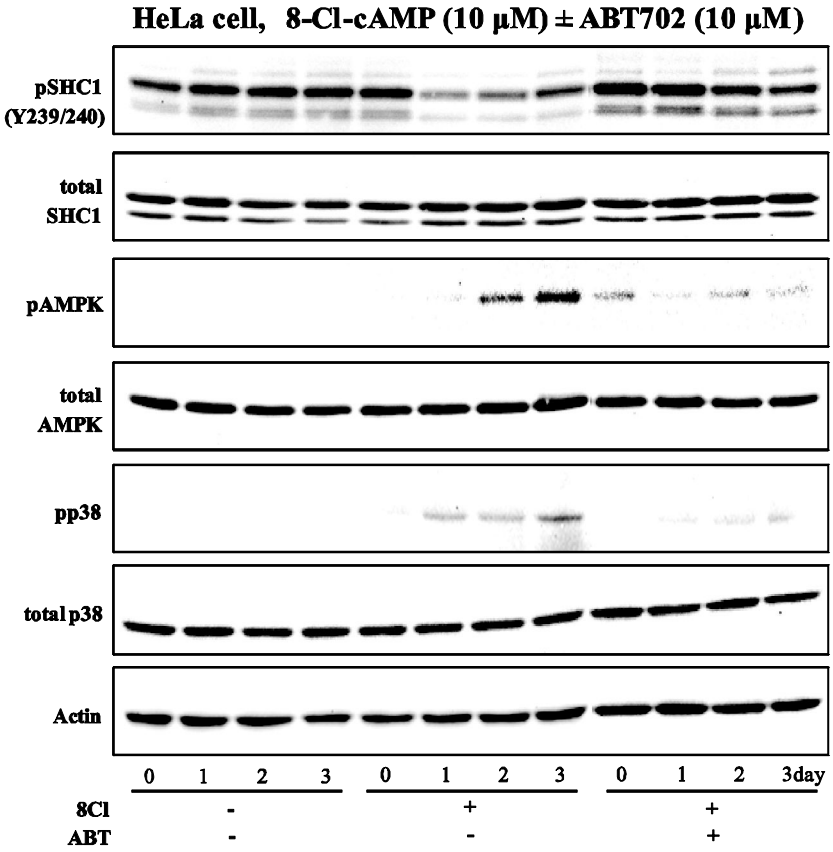

A.
HeLa cell, 8-Cl-Adenosine (5 μM)
pSHC1
(Y239/240)
total SHC1
pAMPK
total
AMPK
pp38
total p38
0 1 2 3 0 1 2 3 day
8Cl-Ado
-
+
B.
HeLa cell, 8-Cl-ATP (5 μM)
pSHC1
(Y239/240)
total SHC1
pAMPK
total
AMPK
pp38
total p38
0 1 2 3 0 1 2 3 day
8Cl-ATP
-
+

Figure 22. The treatment of 8-Cl-cAMP resulted in SHC1 de-phosphorylation.

HeLa cells were treated with 8-Cl-cAMP for the indicated times in the presence or absence of ABT702 (an adenosine kinase inhibitor, 10 μM). Western blotting was carried out using anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1), anti-phospho-AMPK (pAMPK), anti-AMPK (total AMPK), anti-phospho-p38 MAPK (pp38), anti-p38 MAPK (total p38) and anti-Actin (Actin) antibodies.

8Cl: 8-Cl-cAMP, ABT: ABT702


HeLa cell, 8-Cl-cAMP (10 μM) ± ABT702 (10 μM)
pSHC1 (Y239/240)
total SHC1
pAMPK
total AMPK
pp38
total p38
Actin
0 1 2 3 0 1 2 3 0 1 2 3day
8Cl - + +
ABT - - +

essential to exert the influence on the phosphorylation and activity of SHC1.

2. The decrease of SHC1 phosphorylation is limited to cancer cells and not non-transformed cells.

By comparison to studies of Akt/PKB, It was decided to check whether the decreased phosphorylation pattern of SHC1 protein by the treatment of 8-Cl-cAMP is a common phenomenon to non-transformed cells. I employed HDF (Human Dermal Fibroblast) cells as a non-transformed cell model and the effects of 8-Cl-cAMP to SHC1 phosphorylation were tested by Western blotting. While the treatment of 8-Cl-cAMP stimulated the decrease of SHC1 phosphorylation in HeLa cells, it did not seem to induce the de-phosphorylation of SHC1 proteins in HDF cells (Fig. 23).

3. The decrease of SHC1 protein levels plays a role in responsiveness to 8-Cl-cAMP-induced growth inhibition.

Because previous reports from our laboratory demonstrated that the activation of AMPK and p38 MAPK was essential for 8-Cl-cAMP-induced growth inhibition of cancer cell (Han et al., 2009), I decided to examine whether the decreased level of phosphorylation in SHC1 protein was involved in the activation of AMPK and p38 MAPK during the 8-Cl-cAMP induced growth inhibition. To mimic the decrease in the level of protein as well as the level of phosphorylation in

SHC1 protein, I employed the siRNA method targeted against *SHC1* protein (*SHC1* siRNA) (Fig. 24A). Using Western blot analysis, I was able to show that both the protein expression and the phosphorylation level of SHC1 were significantly reduced in HeLa cells 24 hrs after the transfection with the *SHC1* siRNA (Fig. 24B). It was then tested whether the transfection with *SHC1* siRNA could affect the phosphorylation of AMPK and p38 MAPK. When HeLa cells were transfected with *SHC1* siRNA, the phosphorylation of AMPK and p38 MAPK were increased, while transfection with *GFP* siRNA as negative control did not induce the phosphorylation of AMPK, p38 MAPK, markedly (Fig. 24B). The decrease of SHC1 expression and phosphorylation level by the transfection with *SHC1* siRNA could induce the phosphorylation of AMPK and p38 MAPK as much as the treatment with 8-Cl-cAMP did in *GFP* siRNA transfected HeLa cells (Fig. 24B, center three lanes).

I also decided to use the cell counting assay to determine whether the down-regulation of SHC1 expression as well as the phosphorylation could mimic the growth inhibition of cancer cells by 8-Cl-cAMP treatment. The transfection of *SHC1* siRNA could induce the growth inhibition in HeLa cells as well as in MCF7 cells, which is shown in Fig. 25. These results roughly demonstrate that the growth inhibition in cancer cells induced by 8-Cl-cAMP treatment was indeed mediated through the alterations in the level of the phosphorylation of SHC1 protein, because the treatment of 8-Cl-cAMP induced the decrease of only SHC1 phosphorylation, but not the level of protein expression (Fig.20A, 20B).

Figure 23. The decrease of SHC1 phosphorylation by the treatment with 8-Cl-cAMP is limited to cancer cells.

HeLa cells and HDF cells were treated with 8-Cl-cAMP for the indicated times. Western blotting was carried out using anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1) and anti-actin (Actin) antibodies.

8Cl: 8-Cl-cAMP

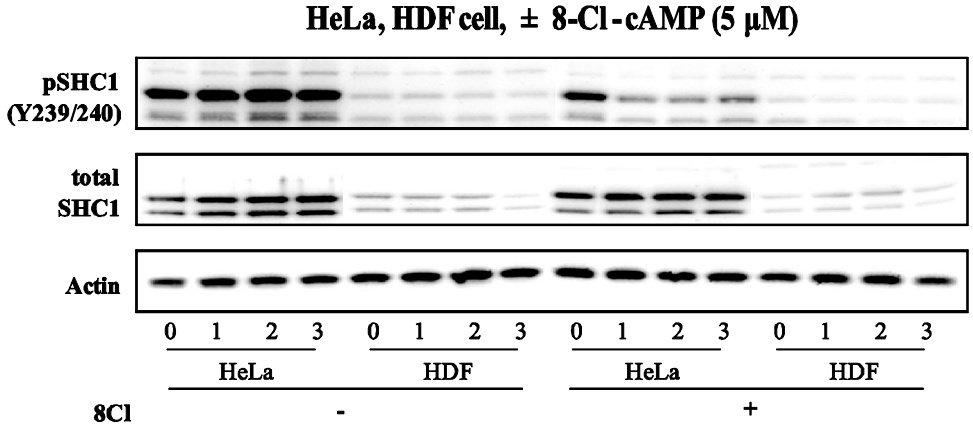

HeLa, HDF cell, ± 8-Cl-cAMP (5 μM)
pSHC1
(Y239/240)
total
SHC1
Actin
0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3
HeLa
HDF
HeLa
HDF
8Cl
-
+

Figure 24. The transfection with *SHC1* siRNA could affect the phosphorylation of AMPK and p38 MAPK.

A. The RNA sequences of the *SHC1*-targeted siRNAs are shown. ***B.*** siRNA targeted against *SHC1* induces the phosphorylation of AMPK and p38 MAPK. *GFP* siRNA transfected HeLa cells were treated with 8-Cl-cAMP (5 μM) for the indicated times 24 hrs after transfection with *GFP*-targeted siRNA (60 nM) and *SHC1*-targeted siRNA (60 nM). Western blotting was performed with anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1), anti-phospho-AMPK (pAMPK), anti-AMPK (total AMPK), anti-phospho-p38 MAPK (pp38) and anti-p38 (total p38) antibodies.

8Cl: 8-Cl-cAMP

A. Sequences of siRNAs targeted to *SHC1*

→ ***SHC1*** **: targeted siRNA of *SHC1***

SHC1 **sense : UGC CAA AGA CCC UGU GAA UdTdT**

SHC1 **antisense : AUU CAC AGG GUC UUU GGC AdTdT**

B.

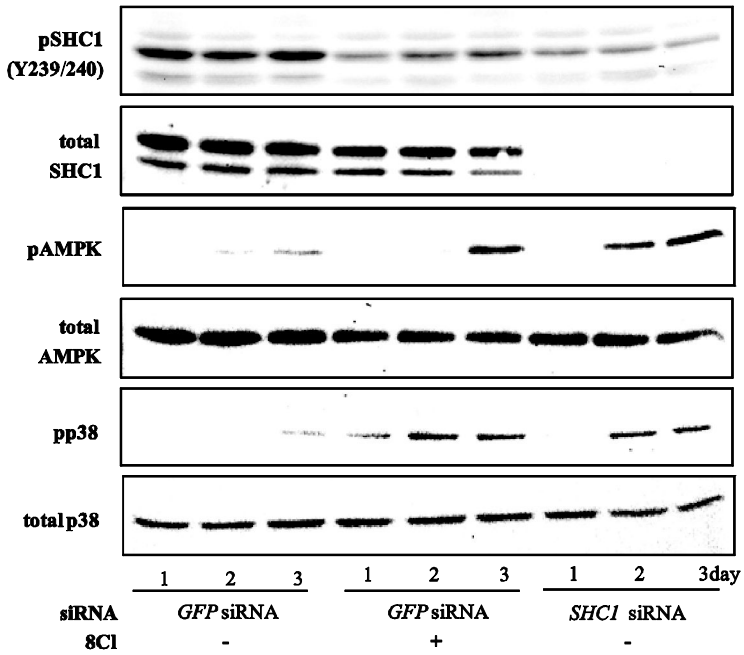
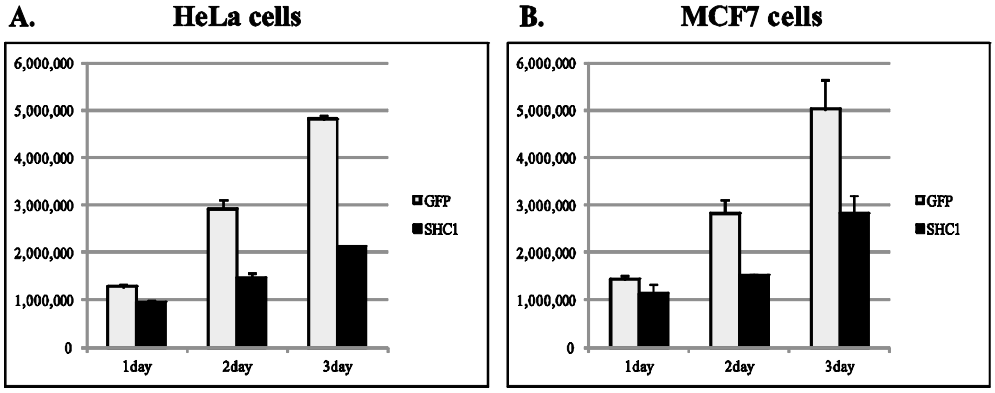
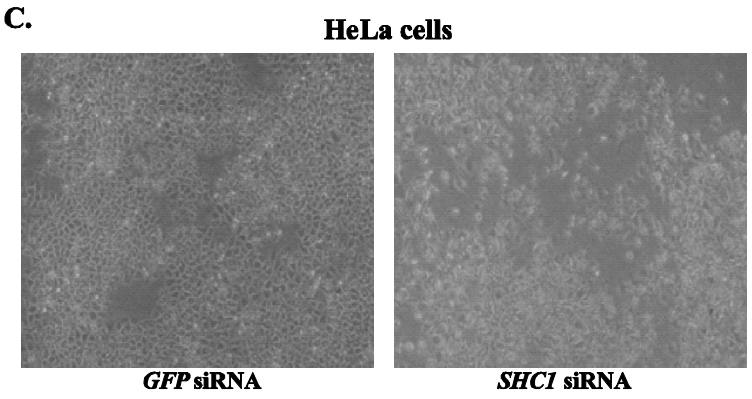


Figure 25. The transfection with *SHC1* siRNA induces the growth inhibition in cancer cells.

A, B. HeLa cells and MCF7cells were incubated for 3 days after the transfection with *SHC1* and *GFP* targeted siRNA, and cells were counted using a Coulter^{TM} counter. Bars denote cell number (mean±SD, n=9). ***C, D.*** HeLa cells and MCF7cells were incubated for 3 days after the transfection with *SHC1* and *GFP* targeted siRNA, and then the pictures were taken using Optiphot-2 fluorescence microscope (Nikon, Tokyo, Japan). Pictures were taken at 40x magnification.

GFP: *GFP* siRNA, SHC1: *SHC1* siRNA


A.
HeLa cells
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
1day
2day
3day
GFP
SHC1
B.
MCF7 cells
6,000,000
5,000,000
4,000,000
3,000,000
2,000,000
1,000,000
0
1day
2day
3day
GFP
SHC1


C.
HeLa cells
GFP siRNA
SHC1 siRNA
D.
MCF7 cells
GFP siRNA
SHC1 siRNA

To clarify the working order of these signaling molecules, I used some chemical inhibitors, i.e., Compound C, the selective AMPK inhibitor, and SB203580, the p38 MAPK specific inhibitor. The phosphorylation of p38 MAPK was decreased by the treatment with Compound C, whereas the decrease of SHC1 phosphorylation was not attenuated during the 8-Cl-cAMP-induced inhibition of cellular growth in HeLa cells (Fig. 26A). While the phosphorylation of MAPKAPK2, downstream effector of p38 MAPK, was effectively blocked by SB203580 treatment, neither the phosphorylation of AMPK nor the de-phosphorylation of SHC1 protein were affected in 8-Cl-cAMP-treated cells (Fig. 26B). The phospho-MAPKAPK2 antibody was employed to measuring kinase activity of p38 MAPK. These data indicate that AMPK may be working in between SHC1 and p38 MAPK in this 8-Cl-cAMP started signaling cascade, i.e. SHC1 on top, AMPK in middle and p38 MAPK at the downstream level, among the three.

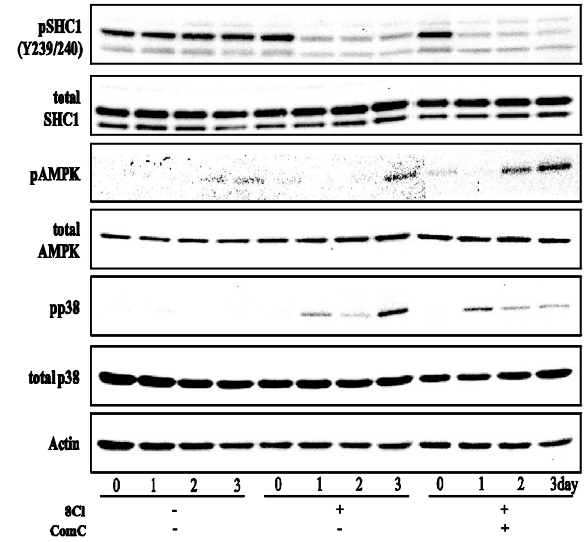
These results clearly demonstrated that SHC1 protein is involved in the phosphorylation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells initiated by 8-Cl-cAMP treatment.

Figure 26. SHC1 acted upstream of AMPK and p38 MAPK during 8-Cl-cAMP-induced growth inhibition.

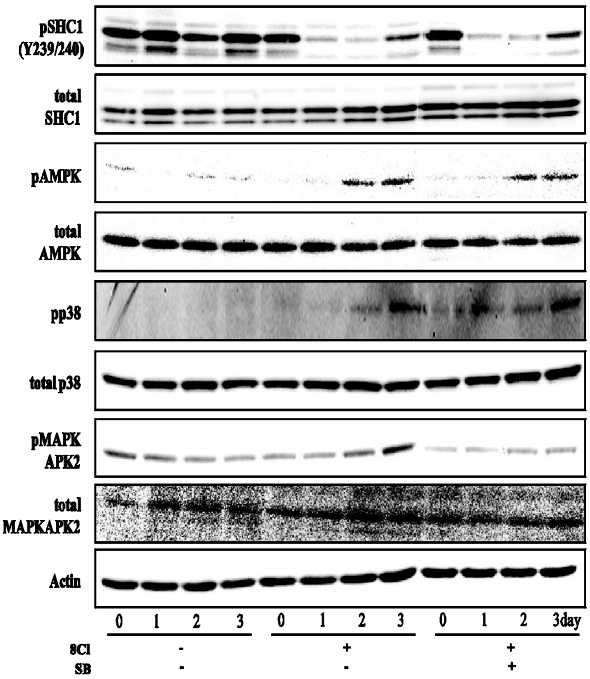
A. HeLa cells were pre-treated with Compound C (an AMPK specific inhibitor, 1 μM) for 1 hr prior to 8-Cl-cAMP (10 μM) for 3 days. Western blotting was performed with anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1), anti-phospho-AMPK (pAMPK), anti-AMPK (total AMPK), anti-phospho-p38 MAPK (pp38), anti-p38 (total p38) and anti-actin (Actin) antibodies. ***B.*** HeLa cells were pre-treated with SB203580 (a p38 MAPK specific inhibitor, 1 μM) for 1 hr prior to 8-Cl-cAMP (10 μM) for 3 days. Western blotting was performed with anti-phospho-SHC1 (pSHC1 (Y239/240)), anti-SHC1 (total SHC1), anti-phospho-AMPK (pAMPK), anti-AMPK (total AMPK), anti-phospho-p38 MAPK (pp38), anti-p38 (total p38), anti-phospho-MAPKAPK2 (pMAPKAPK2), anti-MAPKAPK2 (total MAPKAPK2) and anti-actin (Actin) antibodies.

8Cl: 8-Cl-cAMP, ComC: Compound C, SB: SB203580

A. HeLa cell, 8-Cl-cAMP (10 μM) ± Compound C (1 μM)



B. HeLa cell, 8-Cl-cAMP (10 μM) ± SB203580 (1 μM)



DISCUSSION

8-Cl-cAMP has been studied for its potential as anti-cancer therapeutic agent since it induced cancer cell selective growth inhibition and apoptosis, while it had no effect on non-transformed cell lines (Cho-Chung et al., 1991; Ciardiello et al., 1990; Kim et al., 2001). The name "physiological therapeutic agent" instead of the "chemo therapeutic agent" caught attention of many physicians due to its low cytotoxicity in normal, differentiated cells. However, the detailed action 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 related to physiological conditions. Especially, Akt2/PKBβ among the three Akt/PKB isoforms has been shown to regulate not only cell proliferation and survival (Chen et al., 2012; Fischer-Posovszky et al., 2012; Huang et al., 2011), 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) as well as osteoblast differentiation (Mukherjee et al., 2010). However, little has been reported about the involvement of Akt/PKB activation in cancer cell growth inhibition and/or apoptosis.

Our previous studies showed that the activation of AMPK and p38 MAPK is indispensable for the inhibition of cellular growth as well as cell death in various cancer cells during the treatment of 8-Cl-cAMP and AICAR (Han et al., 2009). In this thesis, I utilized various experimental tools such as Western blot analysis, PI staining and cell counting analysis in combination with the use of specific inhibitors for Akt/PKB, AMPK and p38 MAPK as well as the *Akt2/PKBβ*-targeted siRNA. I showed that the activation of AMPK and p38 MAPK are actually mediated through the activation of Akt/PKB during the treatment with 8-Cl-cAMP and AICAR. The data pointed to the direction that the activation of Akt/PKB can act as one of the upstream trigger factors in 8-Cl-cAMP-induced cancer cell growth inhibition and cell death. It was also shown in Figure 9 that the anti-proliferative activity of 8-Cl-cAMP is limited to cancer cells but not in non-transformed HDF cells and that the Akt/PKB as well as the downstream kinases AMPK and p38 MAPK are not activated in this non-transformed cell line by the treatment of 8-Cl-cAMP. These results obtained with the HDF cell line may serve as the clue for why the growth inhibitory activity of 8-Cl-cAMP is confined only to the cancer cells but not to the non-transformed counterpart.

The use of specific inhibitors, i.e., 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 I did not include the data in this thesis, Rapamycin, an mTOR (a known downstream effector of Akt/PKB)

inhibitor, could not attenuate the phosphorylation of AMPK and p38 MAPK induced by the 8-Cl-cAMP treatment. Also, the overall cell growth inhibition induced by 8-Cl-cAMP treatment was not affected by the treatment with LY294002 and Rapamycin (Figs. 12B, 12C), either. The employment of *Akt/PKB*-targeted siRNA clearly defined the involvement of Akt2/PKBβ but not as much of Akt1/PKBα in the phosphorylation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells by 8-Cl-cAMP treatment (Figs. 13, 14).

Overall, these data point to the direction that the inhibition of cancer cell proliferation by 8-Cl-cAMP is mediated through the Akt2/PKBβ signal transduction pathway, which seems to be PI3K-independent. In the conventional Akt/PKB signaling pathway, Akt/PKB was known to be phosphorylated at Thr(308) and Ser(473) residues by PDK1 and PDK2, which are activated by PI3K kinase (Alessi et al., 1996; Alessi et al., 1997). The data obtained through this thesis work show otherwise. It is likely that the treatment with 8-Cl-cAMP induced the activation of Akt2/PKBβ either directly or through a PI3K-independent, unknown signaling pathway. And mTOR, the downstream effector of Akt/PKB, is not involved in the 8-Cl-cAMP-mediated growth inhibition and cell death in HeLa cells.

Up until now, Akt2/PKBβ has been known to be involved mainly in cell proliferation and survival through the suppression of growth inhibition and apoptosis (Chen et al., 2012; Fischer-Posovszky et al., 2012; Huang et al., 2011). However, this thesis work shows the unusual involvement of Akt2/PKBβ in that the treatment

of anti-cancer drug induces the activation of Akt2/PKBβ and this activated Akt2/PKBβ somehow directs the growth inhibitory effect of the 8-Cl-cAMP in cancer cells. How did the phosphorylation of Akt2/PKBβ by 8-Cl-cAMP induce the cancer cell growth inhibition, in contrast with the phosphorylation of Akt/PKB by various growth factors? The answers are not uncertain. It would be safe to guess that the interaction partners of Akt/PKB by PI3K-independent signaling pathway may differ from the partners by PI3K-dependent signaling pathway. The difference in the interaction partners of Akt/PKB may then affect the regulation of this cancer cell growth. Another research group reported a 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). Accordingly, it is plausible to 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 this is the case, Akt2/PKBβ can become the novel anti-cancer target for the control of cancer cell proliferation.

One remaining question 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, while both chemicals exert the same growth inhibitory effect in a long time frame. So far, I do not have the solid answers. But intelligent guess may be that AICAR activates Akt2/PKBβ

and AMPK physically or directly, whereas the action of 8-Cl-cAMP may be indirect or 8-Cl-cAMP converts into its metabolites first, which takes time. The report by Lucchi et al (2011) claimed that the treatment with 8-Cl-cAMP and AICAR induced apoptosis of various cancer cells through the activation of AMPK and p38 MAPK. They also showed that while the activation of p38 MAPK by 8-Cl-cAMP took more than 24 hrs, the activation of Erk MAPK by 8-Cl-cAMP was achieved within 5mins of treatment. Hence, the existence of quick activation of Erk MAPK by 8-Cl-cAMP could support the possible existence of another early activated signaling pathway for the activation of Akt2/PKBβ, AMPK and p38 MAPK in the processes of cancer cell growth inhibition.

In summary, It was demonstrated with reasonable experimental data 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 thesis, I can clearly establish the order of action for the 3 signaling molecules involved, i.e., Akt/PKB working in the most upstream, AMPK in the middle and p38 MAPK at the downstream (Fig. 27).

I also showed the evidences that the SHC1 protein works as upstream factor of AMPK and p38 MAPK activation in 8-Cl-cAMP-induced cancer cell growth inhibition using the immunoprecipitation assay using antibodies against AMPK and p38 MAPK (Table 1). SHC1 protein was identified as an adaptor protein containing SH2 domains that are conserved regions among a group of cytoplasmic signaling

proteins (Pelicci et al., 1992; Ravichandran, 2001). SHC1 proteins are known to be involved in the signaling of various cell surface receptors such as growth factor receptors (Gelderloos et al., 1998; Pelicci et al., 1992; Pronk et al., 1994), cytokine receptors (Pratt et al., 1996; Velazquez et al., 2000) and hormone receptors (Kousteni et al., 2001; Morte et al., 1998). I expected that the treatment with 8-Cl-cAMP would decrease the phosphorylation of the SHC1 protein as well as its activation, because activated SHC1 was known to stimulate the cell proliferation and metastasis in many cancer cells (Benetti et al., 2004; Du et al., 2013; Ma et al., 2010). First body of work on this aspect showed that the phosphorylation of SHC1 was diminished upon treatment with 8-Cl-cAMP in HeLa and MCF7 cells (Fig. 20).

In this thesis work, it was shown that the activation of AMPK and p38 MAPK are mediated through the decrease of SHC1 phosphorylation during the treatment with 8-Cl-cAMP. The possibility is that SHC1 protein can act as one of the upstream factors of AMPK and p38 MAPK activation in 8-Cl-cAMP-induced cancer cell growth inhibition and cell death. Though I was not able to show the relation between Akt/PKB and SHC1 yet, both Akt/PKB and SHC1 proteins seem to be involved in the activation of AMPK and p38 MAPK in 8-Cl-cAMP-induced cancer cell growth inhibition. Similar to Akt/PKB, the phosphorylation of SHC1 was not decreased by 8-Cl-cAMP treatment in non-transformed cell lines, whereas its phosphorylation was decreased in cancer cell or transformed cell lines (Fig. 23).

The use of siRNA directed against the SHC1 protein clearly shows the

involvement of SHC1 protein in the activation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells and MCF7 cells (Figs. 24, 25). However, it may be necessary to find a way to reduce only the phosphorylation of SHC1, but not the expression level of the SHC1 protein to mimic the effect of 8-Cl-cAMP treatment. With regard to the use of several chemical inhibitors related to the signaling molecules in this study, the phosphorylation of SHC1 protein was not affected by the treatment with Compound C (an AMPK inhibitor) and SB203580 (a p38 MAPK inhibitor) (Fig. 26). These results also support the idea that SHC1 protein is involved in the phosphorylation of AMPK and p38 MAPK and subsequent growth inhibition of HeLa cells induced by 8-Cl-cAMP (Fig. 27).

In this thesis work, I presented the plausible data that both Akt2/PKBβ and SHC1 proteins were involved in 8-Cl-cAMP-induced cancer cell growth inhibition as upstream trigger factors for the activation of AMPK and p38 MAPK. Currently, attempts are being made to identify the missing intermediate molecules other than the Akt2/PKBβ, SHC1, AMPK and p38 MAPK pursued in this study to complete the signaling cascade in this 8-Cl-cAMP induced growth inhibition and/or cell death in cancer cells. The summary of this thesis work is depicted in Figure 27.

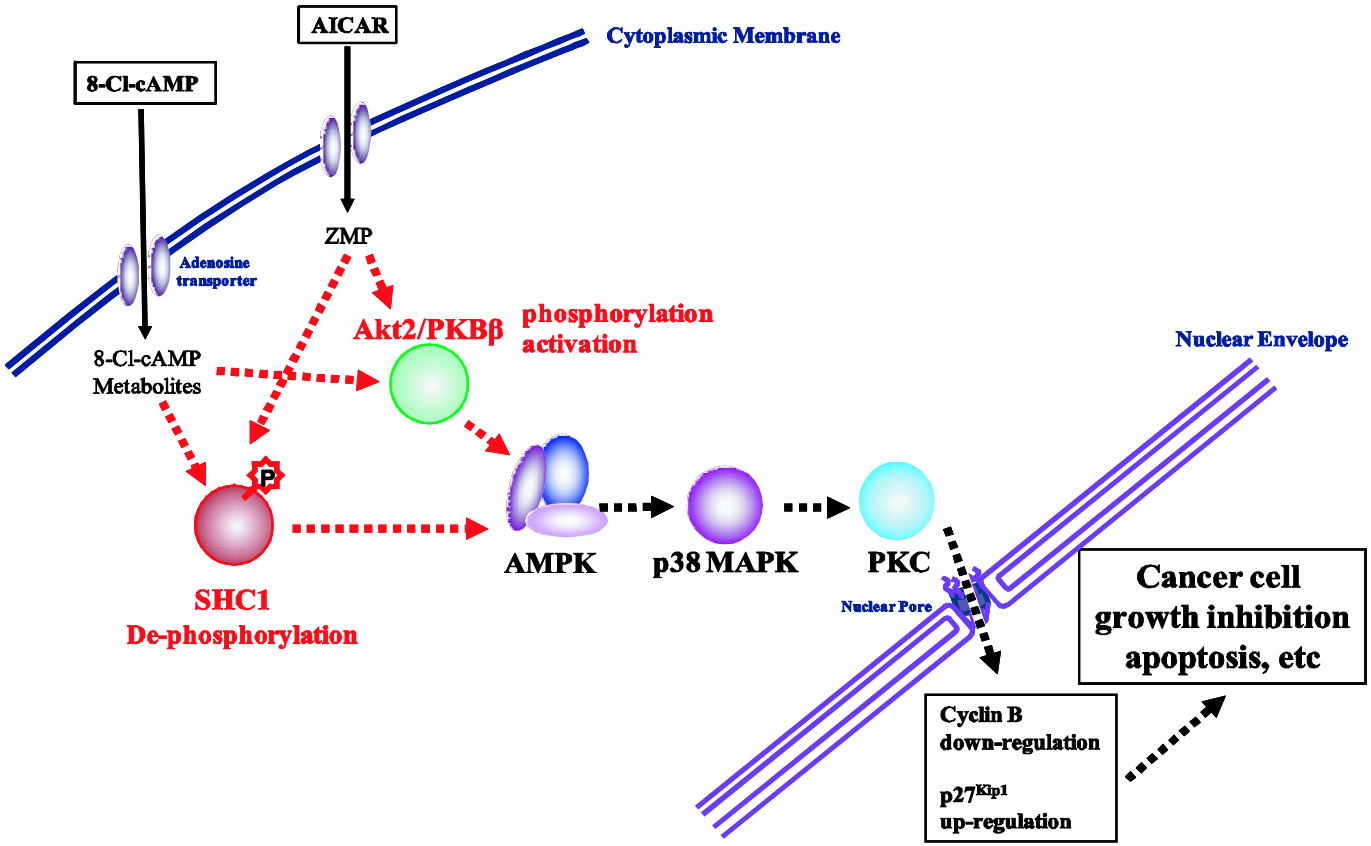
One interesting point to note is that the SHC1 protein, which is known to be over-expressed only in transformed cancer cells, exhibited neither the hyper-expression nor the hyper-phosphorylation in non-transformed counterparts. Combining this with the facts that the treatment of 8-Cl-cAMP promotes the

decrease of SHC1 phosphorylation only in transformed cells, and the anti-proliferative activity of 8-Cl-cAMP is limited to cancer or transformed cell lines suggest us a fresh insight. Could this difference in the level of SHC1 protein in different cell types be the critically important factor dividing the responsiveness of 8-Cl-cAMP in between the cancer and non-transformed counterparts? Ideas are being formulated to test this possibility. Perhaps, modulating the activities of SHC1 proteins in both the cancer and non-transformed cells by the over-expression and/or down-regulation of this gene could give us the first clue.

Figure 27. The summary of Akt/PKB and SHC1 signaling pathway in 8-Cl-cAMP-induced cancer cell growth inhibition

Akt2/PKBβ and SHC1 proteins were involved in 8-Cl-cAMP-induced cancer cell growth inhibition as upstream factors for the activation of AMPK and p38 MAPK. The relationship between the Akt2/PKBβ and SHC1 is yet to be analyzed.

Summary


AICAR
Cytoplasmic Membrane
8-Cl-cAMP
ZMP
Adenosine transporter
8-Cl-cAMP Metabolites
Akt2/PKBβ
phosphorylation activation
Nuclear Envelope
P
AMPK
p38 MAPK
PKC
SHC1
De-phosphorylation
Nuclear Pore
Cancer cell growth inhibition apoptosis, etc
Cyclin B down-regulation
p27Kip1 up-regulation

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국 문 초 록

8-Cl-cAMP가 유도하는 암세포의 세포성장억제 과정에서 작용하는 Akt2와 SHC1 단백질의 기능에 관한 연구

Cyclic AMP는 secondary messenger로서 세포의 성장과 죽음을 비롯하여 다양한 세포 생리학적 기능을 조절하고 있다. 8-chloro-cyclic AMP (8-Cl-cAMP)는 다양한 암세포의 성장억제, 세포사멸, 분화, 암세포 역분화를 유도하는 cAMP analog 중의 하나이다. 8-Cl-cAMP는 암세포에서만 성장억제와 세포사멸을 유도하는 것으로 알려져 있지만 아직까지 정확한 신호전달과정은 밝혀지지 않았다. 따라서 많은 연구자들은 8-Cl-cAMP를 처리하였을 때 암세포에서만 일어나는 것으로 알려진 성장억제와 세포사멸과정의 신호전달과정을 찾는데 많은 노력을 기울여 왔다. 여러 연구 결과들을 통하여 8-Cl-cAMP는 PKA단백질의 RI 서브유닛의 down-regulation을 촉진하고 PKC, Rap1, AMPK 그리고 p38 MAPK의 활성화를 유도하여 암세포의 성장억제와 세포사멸을 유도한다는 사실을 알았다. 또한 8-Cl-cAMP가 8-Cl-adenosine으로 변환되는 것이

세포성장억제 효과를 위한 필수적인 과정임을 알게 되었다.

나는 Akt/PKB 단백질과 SHC1 단백질이 8-Cl-cAMP가 유도하는 암세포 특이적인 성장억제현상에 관련되어 있음을 이 학위논문의 연구결과들을 통하여 증명하였다.

Akt/PKB 유전자는 serine/threonine-specific protein kinase family에 속하는 세 개의 Akt1, Akt2, Akt3 isoform을 암호화하고 있다. 그리고 이 Akt/PKB 단백질들은 세포사멸과정을 억제함으로써 세포의 생존을 유도하는 것으로 알려져 있다. 이러한 이유로 나는 암세포에 8-Cl-cAMP를 처리하게 되면 Akt/PKB의 인산화와 활성화가 줄어들 것으로 예상하였지만 이러한 예상과는 반대로 암세포에 8-Cl-cAMP를 처리하였을 때 Akt/PKB의 인산화가 증가하는 현상을 발견하게 되었다. Adenosine kinase의 억제제인 ABT702와 adenosine transporter 억제제인 NBTI를 암세포에 미리 처리하였을 때 8-Cl-cAMP의 처리에 의한 Akt/PKB의 인산화 증가는 억제되었다. 게다가 AMPK의 억제제인 Compound C, p38 MAPK의 억제제인 SB203580의 처리와 AMPK-DN 돌연변이의 주입에 의해서는 8-Cl-cAMP의 처리에 의한 Akt/PKB의 인산화 증가가 억제가 되지 않은 반면에 Akt/PKB의 억제제인 TCN, *Akt2/PKBβ*를 타겟으로 하는 siRNA의 처리에 의해서는 8-Cl-cAMP와 AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, AMPK activator)에 처리에 의한 AMPK와

p38 MAPK의 인산화 증가를 억제할 수 있었다. 또한 TCN을 암세포에 미리 처리하였을 때에는 8-Cl-cAMP와 AICAR의 처리에 의하여 나타나는 암세포의 성장억제현상을 원상복구 할 수 있었지만 *Akt1/PKBα*를 타겟으로 하는 siRNA를 처리하였을 때에는 8-Cl-cAMP의 처리에 의한 AMPK와 p38 MAPK의 인산화 증가를 막을 수 없었다. 나는 얻어낸 결과들을 통하여 8-Cl-cAMP와 AICAR를 처리하였을 때 Akt2/PKBβ의 인산화 증가가 유도되고 그 결과로 AMPK와 p38 MAPK의 활성화가 촉진된다는 결과를 얻을 수 있었다.

SHC1 단백질은 SH2 도메인을 가지고 있는 adaptor 단백질에 속하는 p46, p52, p66 세가지 isoform으로 발현되는데 각각의 isoform들은 세포반응과정에서 각자 다른 기능들을 유도하는 것으로 알려져 있다. 나는 암세포에 8-Cl-cAMP를 처리하였을 때 SHC1의 인산화는 감소하지만 SHC1 단백질의 전체 단백질량에는 변화가 없다는 사실을 알아냈다. 게다가 암세포에 AMPK의 억제제인 Compound C와 p38 MAPK의 억제제인 SB203580을 미리 처리하였을 때는 8-Cl-cAMP의 처리에 의한 SHC1 단백질의 인산화 감소현상이 억제되지 않았던 반면에 *SHC1*을 타겟으로 하는 siRNA를 처리해서 SHC1 단백질의 인산화가 줄어드는 현상을 재연하였을 때에는 8-Cl-cAMP를 처리했을 때와 비슷하게 AMPK와 p38 MAPK의 인산화를 촉진하는 것으로 보이는 것을 알아냈다. 나는 이러한

결과들을 통하여 8-Cl-cAMP를 처리하였을 때 나타나는 SHC1 단백질의 인산화 감소현상이 AMPK와 p38 MAPK의 인산화 과정에서 상위인자로서 작용한다는 것을 증명하였다.

주요어 : Akt/PKB, SHC1, 8-Cl-cAMP, 암세포, 세포성장억제

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