Role of cyclic AMP response element-binding protein in cancer cells in response to glucose stress

Shin Myung Kang

Department of Medicine

The Graduate School, Yonsei University

Role of cyclic AMP response element-binding protein in cancer cells in response to glucose stress

Directed by Professor Joon Chang

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Shin Myung Kang

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This certifies that the Doctoral Dissertation of Shin Myung Kang is approved.

Thesis Supervisor : Joon Chang

Thesis Committee Member#1 : Joo Hang Kim

Thesis Committee Member#2 : Se Kyu Kim

Thesis Committee Member#3: Hojoong Kim

Thesis Committee Member#4: Kunhong Kim

The Graduate School
Yonsei University

June 2014

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ABSTRACT

Role of cyclic AMP response element-binding protein in cancer cells in response to glucose stress

Shin Myung Kang

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Joon Chang)

Background: cyclic AMP response element-binding protein (CREB) is a leucine zipper transcription factor that regulates expression of target genes involved in metabolism, transcription, cell cycle, survival, DNA repair, growth control, immune regulation, signaling, transport, and reproduction. However, there are very limited data available regarding the role of CREB in human cancer. CREB mediates energy homeostasis and promotes stress resistance in non-cancerous tissues, which is similar to active reprogramming systems of cancer that enable adaptation and survival under environmental metabolic stress. Thus, it is plausible that CREB plays a role in stress resistance or energy compensation in cancer. This study was conducted to characterize the role of CREB in cancer cells in response to glucose starvation and investigate differentially expressed genes under glucose

deprivation conditions.

Methods: Several human cancer cell lines (A549, H1299, MDA-MB-231, MDA-MB-468, BT474, and SKOV-3) were used in this study. Deprivation of glucose or fetal bovine serum from culture media was used to mimic metabolic stress. Transient transfection with siRNA was performed to test CREB-specific roles. Proliferation under normal culture conditions and cell viability assays under extremely low glucose and FBS-free conditions were conducted to explore the metabolic phenotype of CREB. To investigate cell death mode, assessment of poly(ADP-ribose) polymerase (PARP) cleavage by Western blot and an apoptosis assay using a flow cytometer were performed. And the CREB-specific gene expression signature under glucose deprivation conditions was investigated by microarray experiments and gene set enrichment analysis. Finally, microarray results were validated by quantitative real-time-PCR.

Results: Expression of phosphorylated CREB was increased under glucose-free media conditions. Proliferation of CREB-knockdown cells was significantly suppressed compared with that of control cells under normal media conditions ($p < 0.001$). The viability of CREB-knockdown cells was significantly higher than that of control ones under metabolic stress conditions ($p < 0.001$). Western blot analysis revealed that PARP cleavage induced by glucose deprivation was attenuated by knockdown of CREB. Additionally, apoptotic assay revealed that the number of cells undergoing apoptotic death decreased in response to knockdown of CREB under glucose deprivation conditions. CREB-specific gene expression signatures under glucose deprivation conditions revealed that energy metabolism-related gene sets such as glycolysis/gluconeogenesis, oxidative

phosphorylation, and ATP synthesis were highly enriched ($p < 0.00001$). Expression of selected putative CREB target genes such as NDUFV1, ATP5G1, and MVD genes was significantly lower in CREB-knockdown cells than in control cells under glucose deprivation conditions ($p < 0.001$).

Conclusion: CREB is essential for cellular proliferation under normal culture media conditions. Under glucose starvation conditions, CREB is activated and mediates apoptotic cell death in correlation with expression of energy metabolism-related gene sets. These findings provide dynamic role of CREB in cancer cells according to the presence or absence of glucose in culture medium.

Key words : cyclic AMP response element-binding protein, cancer, glucose, stress

Role of cyclic AMP response element-binding protein in cancer cells in response to glucose stress

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I. INTRODUCTION

cyclic AMP response element-binding protein (CREB) is a 43 kDa leucine zipper transcription factor that is ubiquitously expressed in most tissues and conserved from *Drosophila* to humans.[1] CREB is activated by phosphorylation at the serine 133 site in response to diverse signals or kinases such as cAMP, protein kinase A, calcium calmodulin-dependent kinases II, and pp90.[1] Phosphorylated CREB (p-CREB) recruits CREB-binding protein, p300, and transducer of regulated CREB-binding proteins (CRTC) as coactivators at the cAMP-responsive element (CRE), TGACGTCA, and subsequently modulates target gene expression.[1, 2] CREB target genes are involved in a variety of cellular functions including metabolism, transcription, cell cycle, survival, DNA repair, growth control, immune regulation, signaling, transport, and reproduction.[1]

There have been very few investigations of the role of CREB in human cancer. CREB was overexpressed in leukemic blast cells from patients with acute myeloid leukemia compared with normal bone marrow cells.[3-5] Additionally, CREB overexpression was associated with poor prognosis in patients with AML when compared with patients whose marrow did not overexpress CREB.[3] CREB acts as a proto-oncogene in hematopoiesis and plays potential roles in leukemogenesis.[3, 4] Expression of CREB and p-CREB were increased in lung cancer specimens rather than adjacent normal lung tissues.[6] Overexpression of CREB or p-CREB was associated with poor survival in patients with non-small cell lung cancer, especially in those with no smoking exposure.[6] In vitro and in vivo experiments using cancer cells indicated that CREB is involved in migration, invasion and metastasis,[7-10] cell proliferation,[11] and tumorigenesis.[12] Although the CREB pathway modulates one-quarter of the mammalian genome,[2] its role in cancer remains to be further elucidated.

Cancer cells proliferate continually, which ultimately leads to regional and temporal metabolic heterogeneity in growing tumors.[13] Cancer cells in peripheral areas in which nutrients are supplied without limitation proliferate well. Conversely, cells in central areas in which nutrient delivery is limited have to overcome bioenergetic stress or adapt to metabolic stress to survive.[13] To accomplish this, cancer cells develop reprogrammed energy metabolism during tumor development, which is regarded as a hallmark in cancer.[14, 15] Interestingly, several studies of non-cancerous tissues revealed that CREB played roles in energy homeostasis and stress resistance. CREB activity and CRTC recruitment on the peroxisome-proliferation-activated receptor-γ coactivator-1 promoter

contributed to the fundamental bioenergetic compensatory system for recovery from decreased cellular ATP levels by uncoupling agents.[16] *Drosophila* CRTC was activated by starvation.[17] CRTC mutant flies were sensitive to starvation and had s significantly shorter survival time than wild type flies.[17] The CREB/CRTC module was the principal regulator of genes involved in energy homeostasis, mainly in response to fasting.[18-22] Inactivation of the CREB/CRTC module triggered by low nutrients was found to be critical for modulation of lifespan extension.[23] Taken together, these results suggest that CREB promotes stress resistance and compensates for decreased cellular energy level. Notably, responses to metabolic stress modulated by CREB activity appear to be similar to active reprogramming systems that enable cancer to adapt and survive environmental metabolic stress. Thus it is plausible that CREB, an evolutionarily conserved transcriptional factor,[1, 2] plays a role in metabolic stress resistance or energy compensation in cancer. To characterize role of CREB in cancer cells and investigate differentially expressed genes, diverse *in vitro* manipulations including deprivation of glucose or fetal bovine serum (FBS) from culture media were applied in this study to mimic metabolic stress condition *in vivo*.

II. MATERIALS AND METHODS

1. Cell lines and cell culture

Human lung cancer cell lines (A549 and H1299), breast cancer cell lines (MDA-MB-231, MDA-MB-468, and BT474), and an ovarian cancer cell line (SKOV-3) were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics in a humidified incubator containing 5% CO_2 at 37°C. For experiments involving metabolic stress, cells were cultured in RPMI 1640 medium without glucose or FBS for the indicated times.

2. siRNA transfection

Control siRNA and siRNA oligonucleotides targeting CREB were obtained from Sigma-Aldrich. The CREB siRNA sequences were 5′-CAGTGGATAGTGTAACTGA-3′ (#1), 5′-CATTGATTGAGGAGCTAAA-3′ (#2), and 5′-GAAAGAGAGAGGTCCGTCT-3′ (#3). Cells (H1299, A549, and MDA-MB-231) were seeded in 6-well plates at 2 x 10^5 cells per well and then incubated for 24 hours later, after which transient transfections using RNAiMAX (Invitrogen, Grand Island, NY, USA) were conducted with siRNA at a final concentration of 50 nM according to the manufacturer's instructions.

3. Cell proliferation and viability assay

Cell proliferation and viability were determined by counting cells using a trypan blue dye exclusion assay. For the assay, 2 x 10^5 cells were seeded in 6-well plates

and transfected 24 hours later with CREB-targeting or control siRNA. At 48 hours post transfection, cells were cultured in 0.1 mM glucose and FBS-free media or 11 mM glucose- and 10% FBS-containing media. At 48 or 72 hours after the medium was changed, cells were trypsinized, resuspended, and mixed with 1:1 0.4% trypan blue. After incubation for 2 minutes, the stained (dead cells) or unstained (viable cells) cells were counted. Cell viability was expressed as a percentage (number of unstained cells/[number of stained + unstained cells] x 100).

4. Western blot analysis

Cells grown in 6-well plates were harvested and lysed in cold radioimmunoprecipitation assay lysis buffer (50 mM HEPES, pH 7.4; 1% NP-40, 150 mM NaCl, 1 mM EDTA, protease inhibitor and phosphatases inhibitors). Protein concentrations were determined using a BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). A total of 10 μg protein was resolved by 10% SDS-PAGE gels. Separated proteins were transferred to a polyvinylidene difluoride membrane and subsequently blocked with 5% nonfat dry milk and 0.1% Tween 20 in TBS. Immunostaining was then conducted by overnight incubation on the membranes with the following antibodies: anti-CREB (Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated CREB (Cell Signaling Technology, Danvers, MA, USA), anti-phosphorylated AMP-activated protein kinase (Millipore, Billerica, MA, USA), anti-microtubule-associated protein 1 light chain 3B (Cell Signaling Technology, Danvers, MA, USA), anti-poly(ADP-ribose) polymerase (Cell Signaling Technology, Danvers, MA, USA), or anti-actin (Sigma-Aldrich, St. Louis, MO, USA). β-actin was used as loading control.

Membranes were washed 0.1% Tween 20 in TBS and probed with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody, after which the immunoreactive bands were visualized with enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA).

5. Apoptosis assay

A Phycoerythrin Annexin V/7-Amino-Actinomycin (7-AAD) Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's protocol. Briefly, MDA-MB-231 and A549 cells were seeded in 6-well plates for 24 hours, then transfected with CREB-targeting or control siRNA. At 48 hours post transfection, the cells were cultured in glucose-free media or glucose-containing (11mM glucose) media. At 48 hours after culture medium change, cells were harvested in annexin-binding buffer and stained simultaneously with FITC-annexin V and 7-AAD. Finally, cells were analyzed using a Becton Dickinson flow cytometer.

6. RNA isolation, microarray experiment, and data analysis

Total RNA was isolated from the H1299 cell line harvested after each treatment using a *mir*Vana™ miRNA Isolation Kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol. Biotin-labeled cRNA samples were prepared using an Illumina TotalPrep RNA Amplification Kit (Ambion Inc., Austin, TX, USA). A total of 500 ng of RNA was used for labeling and hybridization as recommended by the manufacturer. A total of 1.5 μg of biotinylated cRNA per sample was hybridized to an Illumina Human-6 BeadChip

v.2 microarray, and signals were developed using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Data were analyzed using Illumina Bead Studio v3.0 after scanning with an Illumina Bead Array Reader confocal scanner (BeadStation 500GXDW; Illumina Inc.). BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) were used for statistical analysis of gene expression data. The value of each sample was normalized by a quantile normalization method and a random-variance *t*-test was applied to calculate the significance of each gene for comparisons of two classes. Gene Set Enrichment Analysis (GSEA) was performed against the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Kolmogorov-Smirnov statistic was applied for evaluation of the statistical significance of each category.

7. Quantitative real-time PCR

Total RNA was purified using the PureLink® RNA Mini Kit (Invitrogen, Grand Island, NY, USA), while quantitative real-time-PCR (qRT-PCR) was performed using the iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Equal amounts of total RNA (15 ng for each sample) were mixed, and target genes were amplified with a specific primer set using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primers sequences used are as follows (Sigma-Aldrich, St. Louis, MO, USA): NADH dehydrogenase ubiquinone flavoprotein 1 (*NDUFV1*) forward: 5′-ATGAAGGTGACAGCGTGAGG-3′; *NDUFV1* reverse: 5′-TTTTCTTGGGTGCTGTCCGC-3′; ATP synthase, H^+ transporting, mitochondrial Fo complex, subunit C1 (*ATP5G1*) forward: 5′-

GGCTAAAGCTGGGAGACTGA-3′; *ATP5G1* reverse: 5′-CAGGCCTGATTAGACCCCTG-3′; mevalonate decarboxylase (*MVD*) forward: 5′-GCGGTCATCAAGTACTGGGG-3′; *MVD* reverse: 5′-GGTCCTCGGTGAAGTCCTTG-3′; 7SL scRNA forward: 5′-ATCGGGTGTCCGCACTAAGTT-3′; 7SL scRNA reverse: 5′-CAGCACGGGAGTTTTGACCT-3′. 7SL scRNA levels were used as an endogenous control. The qRT-PCR data were calculated using the comparative threshold cycle method and the iCycler CFX96 analyzer software.

8. Statistical analysis

Data are representative of at least two independent experiments. Cell proliferation and cell viability were compared by a two-tailed unpaired Student's *t* test and data were presented as means ± the standard deviation (SD). Analyses were performed using an SPSS package, network version 17.0 (SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered significant.

III. RESULTS

1. Expression of CREB and phosphorylated CREB under normal culture media conditions

Cells were cultured under normal media conditions to investigate baseline expression status of CREB and p-CREB. Under normal culture media conditions, expression of CREB or p-CREB showed a wide range of levels across cell lines (Fig. 1). MDA-MB-231, MDA-MB-468, and SKOV-3 displayed high levels of expression of CREB or p-CREB, while lung cancer cell lines (A549 and H1299) showed relatively intermediate levels of CREB or p-CREB expression and BT474 showed a very low level of CREB or p-CREB expression.

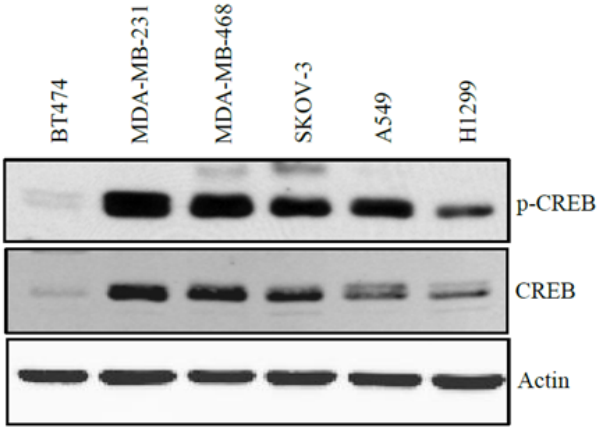

Figure 1. Expression of CREB and p-CREB in cancer cell lines under normal media conditions. Western blot analysis using total cell lysates from breast cancer cell lines (BT474, MDA-MB-231, and MDA-MB-468), an ovarian cancer cell line (SKOV-3), and lung cancer cell lines (A549 and H1299) cultured under normal media conditions.

2. Activation of CREB under glucose-free media conditions

To test whether CREB is activated under metabolic stress conditions, cells were cultured in glucose-free media. Among the cell lines used in the present study, BT474 and H1299, which showed a relatively low level of p-CREB under normal culture media conditions, were selected for reliable detection of further increases in phosphorylation of CREB by Western blot method. Under glucose-free media conditions, expression of p-CREB was subsequently increased in BT474 (Fig. 2). This is in accordance with increased phosphorylation of AMP-activated protein kinase (AMPK), which acts as an intracellular energy sensor.[24] H1299 also showed increased expression of p-CREB at 36 hours after glucose starvation. Taken together, these results indicate that CREB is activated under glucose deprivation conditions.

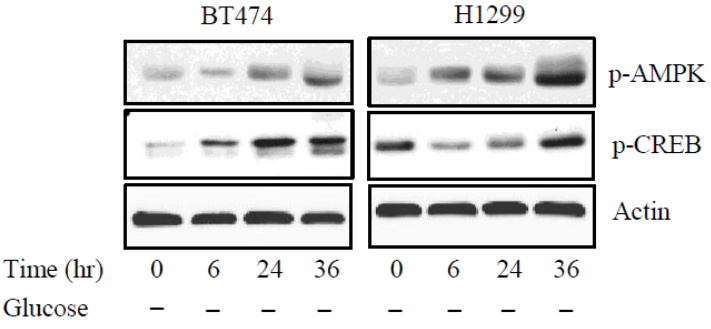

Figure 2. Expression of p-CREB under glucose-free media conditions. Western blot analysis using total cell lysates from BT474 and H1299 cell lines cultured in glucose-free media.

3. Knockdown of CREB with siRNA

Knockdown of CREB with siRNA and modification of culture medium components were conducted to explore the metabolic phenotypic characteristics of CREB in cancer. Efficient knockdown of CREB with three different oligonucleotides was confirmed by Western blot analysis (Fig. 3).

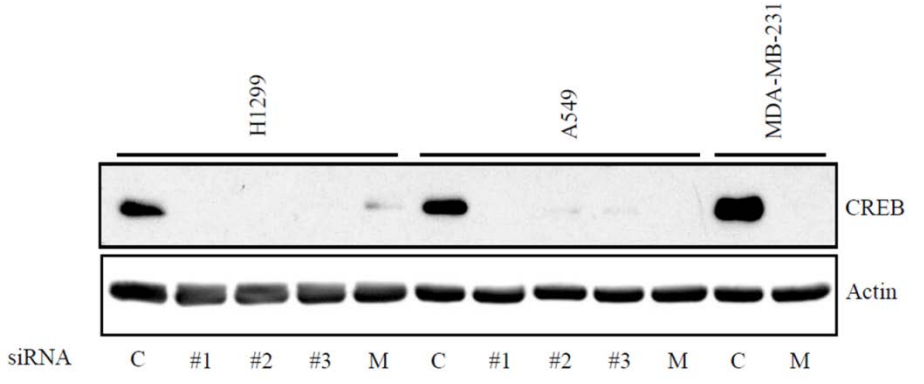

Figure 3. Knockdown of CREB by three siRNA oligonucleotides targeting CREB in transiently transfected cells. Western blot analysis of CREB expression after transient transfection with control siRNA (C), siRNA for CREB (#1 oligonucleotide sequence; 5′-CAGTGGATAGTGTAACTGA-3′, #2 oligonucleotide sequence; 5′-CATTGATTGAGGAGCTAAA-3′, #3 oligonucleotide sequence; 5′-GAAAGAGAGAGGTCCGTCT-3′, and mixture [M] of #1 oligonucleotide, #2 oligonucleotide, and #3 oligonucleotide).

4. Effect of CREB knockdown on cell proliferation under normal culture media conditions

To investigate role of CREB in cancer cell proliferation, cells treated with siRNA for CREB or control were cultured under normal media conditions and compared the cell number. As shown in Figure 4, proliferation of H1299 and A549 treated with CREB siRNA compared with control siRNA were significantly suppressed under normal media conditions, suggesting that CREB may be required for cancer cell proliferation under metabolically nourished conditions.

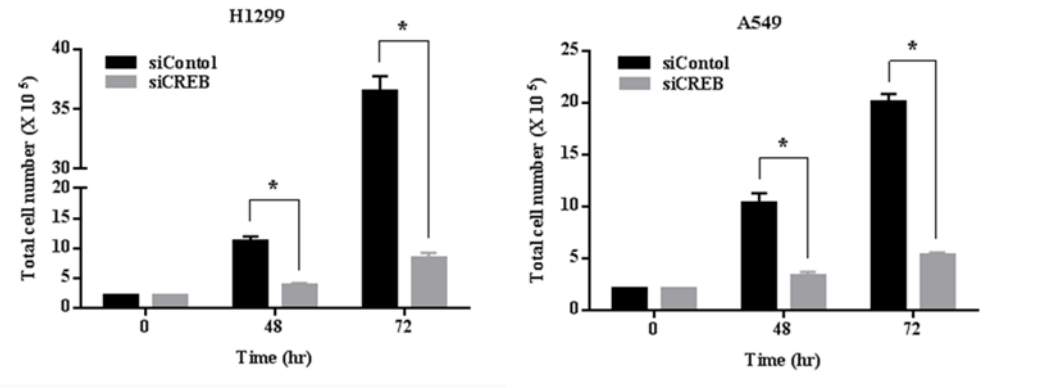

Figure 4. Effect of CREB knockdown on cell proliferation under normal media conditions. Cells (2 x 10^5 cells/well) were seeded and transfected 24 hours later with CREB-targeting or control siRNA. Live cell number after 48 hr or 72 hr of culture under normal media conditions was counted. The data are presented as the mean ± SD of triplicate samples. *, $p < 0.001$

5. Effect of CREB knockdown on cell viability under metabolic stress conditions

To investigate role of CREB in cancer cell survival under metabolic stress condition, cells treated with siRNA for CREB or control were cultured under extremely low glucose (0.1 mM) and FBS-free conditions and compared cell viability. Extremely low glucose and FBS-free culture conditions after treatment of CREB siRNA resulted in decreased cell death when compared with control siRNA treatment across cell lines (Fig. 5).

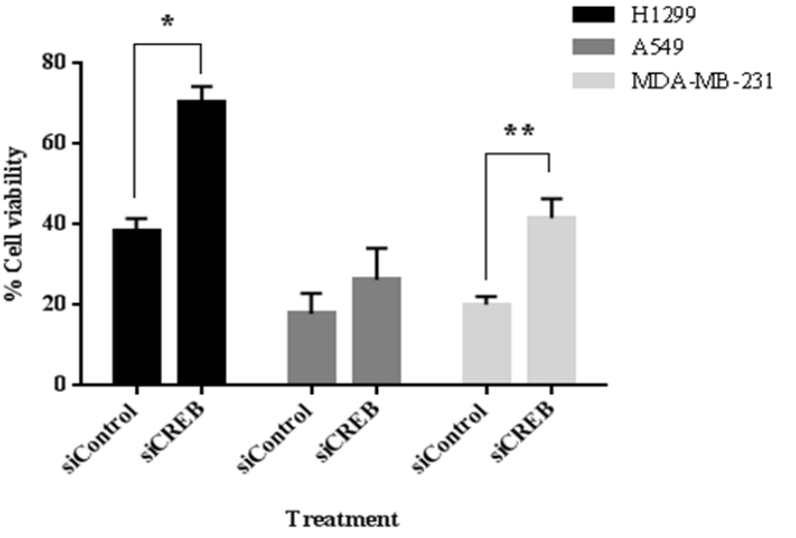

Figure 5. Effect of CREB knockdown on cell viability under metabolic stress conditions. Cells (2 x 10^5 cells/well) were seeded and transfected 24 hours later with CREB-targeting or control siRNA. At 48 hours post transfection, cells were cultured in 0.1 mM glucose and FBS-free media. At 48 hours after the medium was changed, cells were trypsinized, resuspended, and mixed with 1:1 0.4% trypan blue. The number of stained or unstained cells was counted. Cell viability was expressed as a percentage (number of unstained cells/[number of stained + unstained cells] x 100). The data are presented as the mean ± SD of triplicate samples. *, $p < 0.001$; **, $p < 0.05$

6. Mode of cell death mediated by CREB under glucose deprivation conditions

Metabolic stress can induce apoptosis, autophagic cell death, or necrosis.[13, 25] To explore which modes of cell death are associated with CREB under glucose deprivation conditions, apoptosis and autophagic cell death were investigated.

Poly(ADP-ribose) polymerase (PARP) cleavage is a hallmark of apoptosis. In Western blot analysis, PARP cleavage induced by glucose deprivation was attenuated by knockdown of CREB (Fig. 6A). In the apoptotic assay, the number of cells undergoing apoptotic death decreased in response to knockdown of CREB under glucose deprivation conditions (Fig. 6B).

Microtubule-associated protein 1 light chain 3B (LC3B) is processed from a cytosolic full-length form (LC3B-I) to a cleaved membrane-bound form (LC3B-II) during autophagy.[26] Conversion from LC3B-I to LC3B-II was increased in A549 and MDA-MB-231 cells cultured in glucose-free media when compared to those grown in normal growth media (Fig. 7). However, treatment of CREB-targeting siRNA did not induce any difference in conversion from LC3B-I to LC3B-II under glucose-free media conditions relative to control siRNA (Fig. 7).

Taken together, these results suggest that CREB knockdown protects cells from apoptotic cell death induced by glucose deprivation, but not from autophagic cell death.

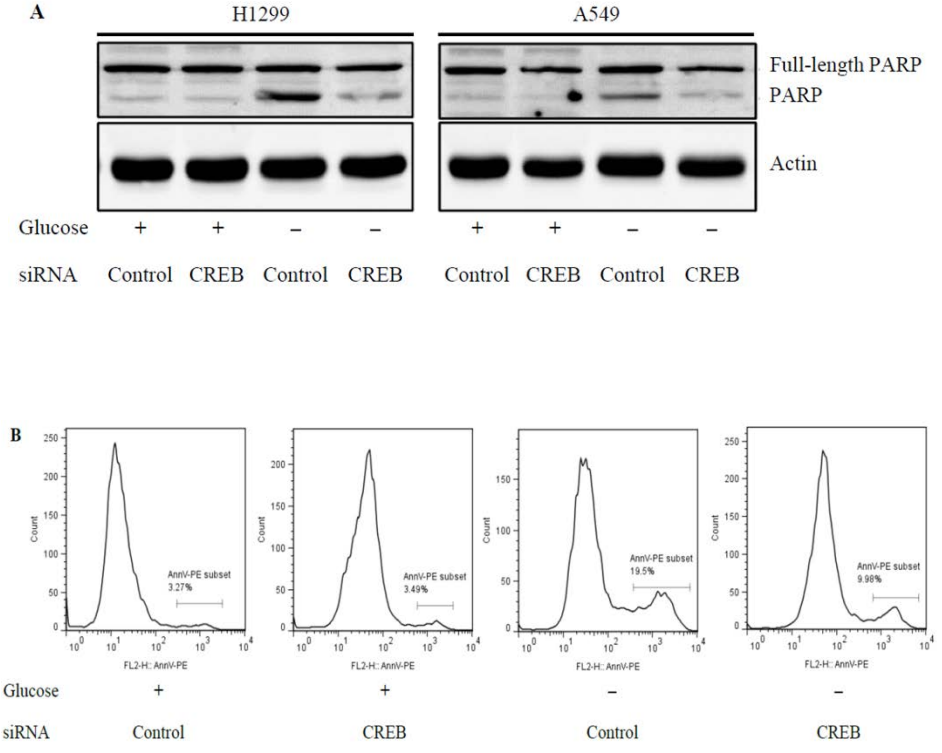

Figure 6. Effect of CREB knockdown on apoptotic cell death under glucose deprivation conditions. PARP cleavage assessed by Western blot (A) and apoptotic cell death assessed by flow cytometry (B). Forty-eight hours after transient transfection with CREB-targeting or control siRNA, cells were cultured in glucose-free or normal media. Cells were harvested at 48 hr after medium changes for preparation of lysates or FITC-annexin V and 7-AAD staining.

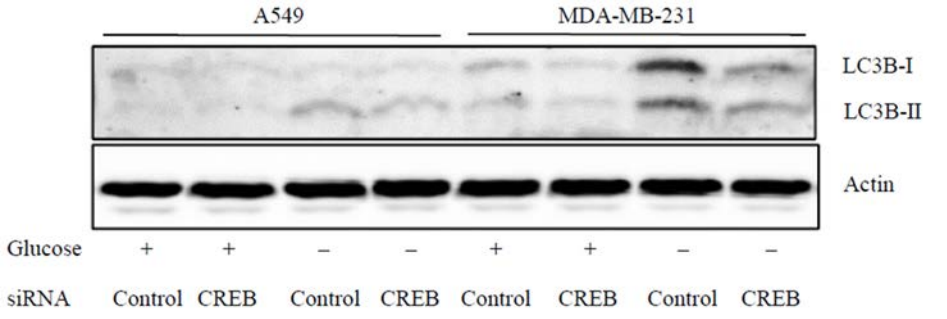

Figure 7. Effect of CREB knockdown on autophagic cell death under glucose deprivation conditions. Western blot analysis to identify conversion from LC3B-I to LC3B-II. Forty-eight hours after transient transfection with CREB-targeting or control siRNAs, cells were cultured in glucose-free or normal media. Lysates were prepared at 48 hr after medium changes.

7. Gene expression signatures under glucose deprivation conditions and gene set enrichment analysis

To identify the CREB-specific gene expression signature under glucose deprivation conditions, gene expression data were generated from H1299 cells treated with siRNA for CREB or control. A two-sample *t*-test was then applied to select genes that were differentially expressed between treatments, which resulted in selection of 27 genes using a statistical cut-off of $p < 0.001$ and 2-fold difference (Fig. 8A). GSEA was then applied to gain biological insight into the differences in gene expression between treatments. GSEA identified 13 gene sets based on a statistical cut-off of $p < 0.00001$ (Table 1). It should be noted that sets of genes involved in energy metabolism such as glycolysis/gluconeogenesis, oxidative phosphorylation, and ATP synthesis were highly enriched (Table 1). The majority of the components of energy metabolism-associated gene sets were downregulated in CREB-knockdown cells when compared with the control under glucose deprivation conditions (Fig. 8B-C).

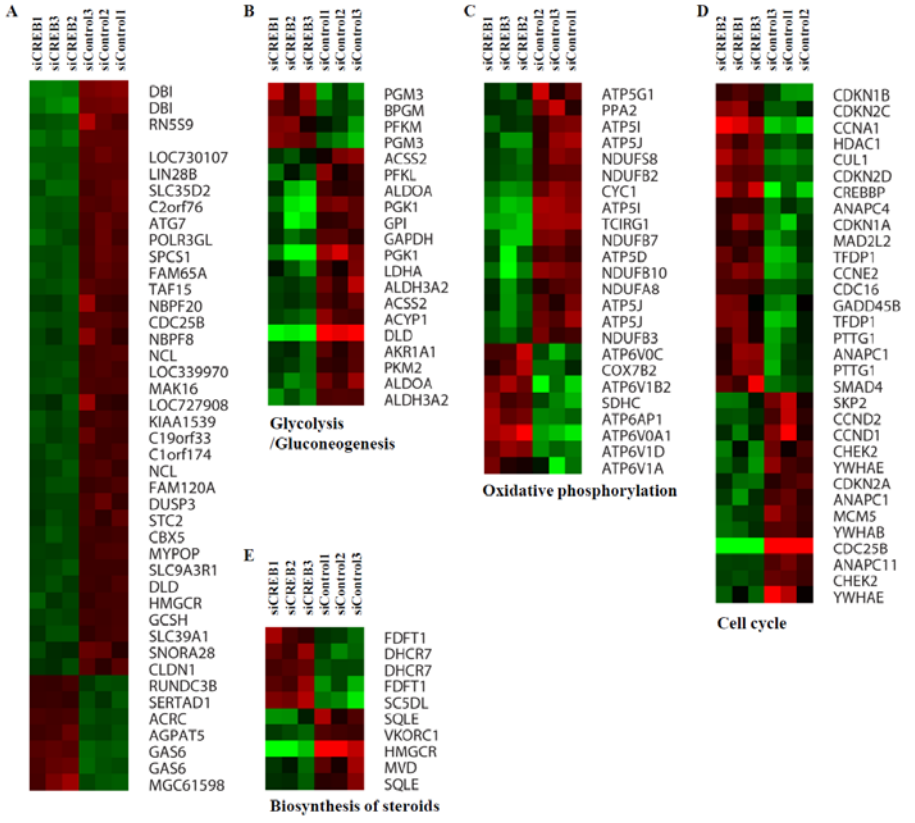

Figure 8. Gene expression signature under glucose deprivation conditions and gene set enrichment analysis. Forty-eight hours after transient transfection with CREB-targeting or control siRNA, H1299 cells were cultured in glucose-free medium. RNA for microarray analysis was isolated at 48 hr after medium changes. A. CREB-specific genes showing significant differences in expression ($p < 0.001$ and 2-fold difference) upon two sample *t*-test analysis are presented as a heat map. The red and green colors reflect high and low relative expression levels, respectively. B-E. CREB-specific gene sets showing differentially expressed pathways ($p < 0.00001$): glycolysis/gluconeogenesis, oxidative phosphorylation, cell cycle, and biosynthesis of steroids, respectively.

Table 1. Lists of differentially expressed pathways by GSEA

KEGG pathway	Pathway description	*p*-value
hsa00010	Glycolysis/gluconeogenesis	< 0.00001
hsa00100	Biosynthesis of steroids	< 0.00001
hsa00190	Oxidative phosphorylation	< 0.00001
hsa00193	ATP synthesis	< 0.00001
hsa00240	Pyrimidine metabolism	< 0.00001
hsa00710	Carbon fixation	< 0.00001
hsa04110	Cell cycle	< 0.00001
hsa04120	Ubiquitin mediated proteolysis	< 0.00001
hsa04310	Wnt signaling pathway	< 0.00001
hsa04510	Focal adhesion	< 0.00001
hsa05120	Epithelial cell signaling in Helicobactor pylori infection	< 0.00001
hsa04910	Insulin signaling pathway	< 0.00001
hsa04350	TGF-beta signaling pathway	< 0.00001

8. Validation of gene expression signatures

To validate the GSEA results, qRT-PCR of selected genes that may play key roles in energy metabolism in cancer was conducted. To select CREB target genes from the enriched gene sets, CREB Target Gene Database was used (http://natural.salk.edu/CREB). Prediction of CREB target genes was based on the presence of CRE sites in promoter regions, which were defined as 3 kilobase upstream to 300 base pairs downstream of the annotated transcription start sites and downstream (within 300 base pairs) TATA boxes.[27] NDUFV1, ATP5G1, and MVD genes that have CRE site and TATA box in their promoters were selected. *NDUFV1* encodes a 51 kDa subunit of the NADH:ubiquinone oxidoreductase electron-transporting protein complex I, while *ATG5G1* encodes a subunit C1 of the mitochondrial ATP synthase and *MVD* catalyzes the conversion of mevalonate pyrophosphate into isopentenyl pyrophosphate in cholesterol biosynthesis. The results of qRT-PCR paralleled the mRNA expression levels acquired via GSEA. Expression of NDUFV1, ATP5G1, or MVD genes of CREB-knockdown cells under glucose deprivation conditions was significantly lower than those of control cells (Fig. 9).

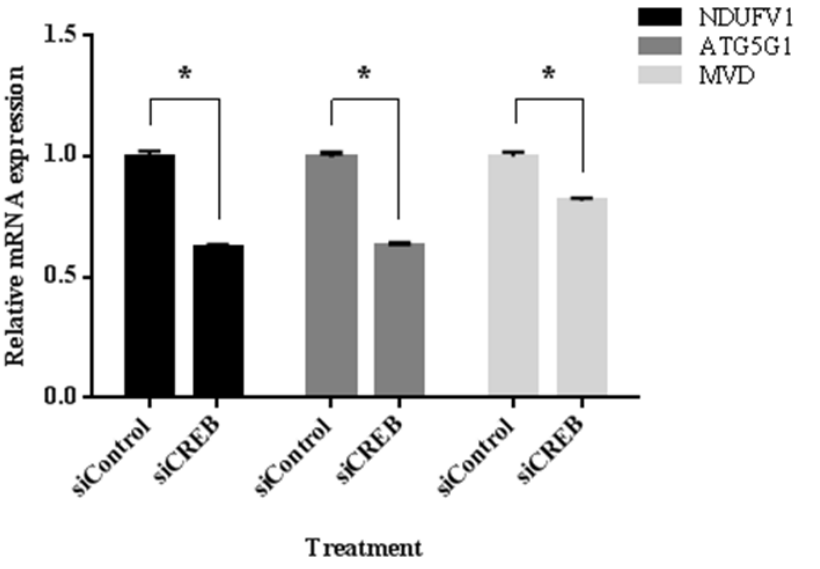

Figure 9. qRT-PCR analysis of putative CREB target genes under glucose deprivation conditions. Forty-eight hours after transient transfection with CREB-targeting or control siRNA, H1299 cells were cultured in glucose-free medium. RNA for qRT-PCR was isolated at 48 hr after medium changes. qRT-PCR was performed to measure relative levels of selected CREB target genes: *NDUFV1*, *ATP5G1*, and *MVD*. The data are presented as the mean ± SD of triplicate samples. *, $p < 0.001$

IV. DISCUSSION

Individual cancer cell undergoes fluctuations in nutrients availability as a tumor grows. Overcoming temporal and regional metabolic challenges is critically important to cancer cell proliferation and survival.[13] Roles of CREB in compensation for decreased cellular energy level and promotion of stress resistance in insulin-sensitive tissues have led to investigation of whether CREB play similar roles in cancer cells.

The results of the present study showed that CREB was activated by glucose starvation. Additionally, GSEA identified high enrichment of gene sets associated with intracellular energy production (glycolysis/gluconeogenesis, oxidative phosphorylation, and ATP synthesis) and upregulation of the majority of their components in cells treated with siRNA for control compared with cells treated with siRNA for CREB under glucose deprivation conditions. This correlation between CREB activation and upregulation of bioenergetics process-related gene sets suggests that CREB may have role in restoration of intracellular energy deficit caused by glucose deprivation.

This study demonstrated that CREB was a critical prerequisite for cellular proliferation in normal culture media and that CREB mediated apoptotic cell death under metabolic stress conditions. A previous investigation showed that human myeloid leukemic cell lines stably overexpressing CREB had higher proliferative activity than parental cells under normal growth conditions,[3] which supports the results of the present experiment. Glucose metabolism has been shown to play an important role in regulation of apoptosis. Glucose deprivation can lead to

apoptotic cell death by inducing an imbalance of antiapoptotic and proapoptotic proteins. Additionally, glucose stress can induce activation of p53,[28] AMPK,[29, 30] Puma,[31] Bim,[32] Noxa,[33] caspase 8,[34, 35] or ER stress,[32] which mediate apoptotic cell death. This study revealed that CREB is another important molecule mediating glucose stress-induced apoptotic cell death. Taken together, these results showed dynamic role of CREB in modulation of cellular proliferation and apoptotic cell death according to the presence or absence of glucose in culture medium.

Previous studies using osteosarcoma-derived U2O2 cells bearing wild-type p53 suggested that transactivation of p53 by CREB contributed to apoptotic cell death under glucose deprivation.[36, 37] However, the H1299 cell line used in this study has a homozygous partial deletion of the p53 gene and therefore does not express the p53 protein. Hence, additional molecule other than p53 or transcriptional mechanism regulated by CREB under glucose deprivation condition remains to be determined in the future study.

This study showed that expression of gene sets associated with energy metabolism was significantly lower in CREB-knockdown cells than control cells under glucose stress conditions. These gene expression profiles do not appear to be correlated with survival advantage of CREB-knockdown cells under metabolic stress since enhancement of the bioenergetic pathway (glycolysis and oxidative phosphorylation) and inhibition of biosynthetic pathway (lipid and protein synthesis or cell cycle progression) are major strategies of metabolic adaptation in cancer.[13-15, 24] Simultaneous regulations of bioenergetic and biosynthetic pathways by CREB may be one potential explanation for the results of this study. GSEA displayed upregulation of cyclin-dependent kinase inhibitor genes (*CDKN1A*,

CKDN1B, *CKDN2C*, and *CDKN2D*), but downregulation of cyclin D genes (*CCND1* and *CCND2*) in CREB-knockdown cells (Fig. 8D). Rate-limiting enzyme for cholesterol biosynthesis (*HMGCR*) was downregulated in CREB-knockdown cells (Fig. 8E). Thus, correlation of gene expression profiles showing inhibition of cell cycle progression and steroid biosynthesis with enhanced cell viability of CREB-knockdown cells under metabolic stress was found. Therefore, it is possible that CREB activated in response to metabolic stress begins to compensate for bioenergetic deficit by upregulating ATP synthesis-related genes, but upregulates high energy-consumption process-related genes such as cell cycle progression and steroid biosynthesis at the same time. Induction of energy-producing and energy-consumption processes simultaneously under limited nutrient supply may disrupt cellular bioenergetic fitness and can lead to apoptotic cell death. However, further studies into the regulatory mechanism of gene expression through which activated CREB under metabolic stress determines cancer cell fate is required.

It should be noted that there are several limitations to this study. First, establishment of stable cell lines overexpressing CREB may be needed to confirm the results of this study. Second, microarray data generated in this study showed gene expression profiles under glucose deprivation conditions only after transient transfections with siRNA. Gene expression profiles under normal growth conditions, combination of four sets of experimental conditions, and comparison of the data may help to uncover the role of CREB by narrowing down target genes or gene sets differentially expressed under metabolic stress conditions. Finally, direct CREB target genes were not clearly identified among the genes differentially expressed under glucose deprivation conditions in this study. To find

direct target genes regulated by CREB under metabolically stressed conditions, identification of physical interactions between CREB and the CRE site of the gene is essential. Further research using a chromatin immunoprecipitation assay or electrophoretic mobility shift assay will be helpful in identification of direct target genes regulated by CREB under metabolically stressed conditions.

In conclusion, CREB is essential for cellular proliferation under normal culture media conditions. Under glucose starvation conditions, CREB is activated and mediates apoptotic cell death in correlation with expression of energy metabolism-related gene sets. These findings provide dynamic role of CREB in cancer cells according to the presence or absence of glucose in culture medium.

V. CONCLUSION

Role of CREB in cancer cells in response to glucose starvation was investigated in this study. In addition, CREB-specific gene expression signature under glucose deprivation conditions was investigated to correlate metabolic phenotype and differentially expressed genes. The results were as follows:

1. Expression of p-CREB was increased under glucose-free media conditions.
2. Proliferation of CREB-knockdown cells was significantly lower than that of control cells under normal media conditions.
3. Viability of CREB-knockdown cells was significantly higher than that of control cells under metabolic stress conditions.
4. PARP cleavage induced by glucose deprivation was attenuated by knockdown of CREB. Apoptotic assay revealed that the number of cells undergoing apoptotic death decreased in response to knockdown of CREB under glucose deprivation conditions. Treatment of CREB-targeting siRNA did not induce any difference in conversion from LC3B-I to LC3B-II under glucose deprivation conditions when compared with control siRNA.
5. CREB-specific gene expression signatures under glucose deprivation conditions revealed 27 differentially expressed genes based on a statistical cut-off of $p < 0.001$ and 2-fold difference. GSEA identified 13 gene sets under a statistical cut-off of $p < 0.00001$. Interestingly, sets of genes involved in energy metabolism such as glycolysis/gluconeogenesis, oxidative phosphorylation, and ATP synthesis were highly enriched.

6. qRT-PCR analysis revealed significantly lower expression of putative CREB target genes such as NDUFV1, ATP5G1, and MVD genes in CREB-knockdown cells compared with control cells under glucose deprivation conditions.

In summary, CREB is essential for cellular proliferation under normal culture media conditions. Under glucose starvation conditions, CREB is activated and mediates apoptotic cell death in correlation with expression of energy metabolism-related gene sets. These findings provide dynamic role of CREB in cancer cells according to the presence or absence of glucose in culture medium.

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ABSTRACT(IN KOREAN)

암세포에서 glucose 스트레스에 대한 cyclic AMP response element-binding protein의 역할

<지도교수 장 준>

연세대학교 대학원 의학과

강 신 명

서론: cyclic AMP response element-binding protein (CREB)는 leucine zipper 모티프 형태의 전사인자이다. CREB은 대사, 전사, 세포주기, DNA 수리, 성장 조절, 면역 조절, 신호전달, 운반, 생식 등에 관련된 유전자 발현을 조절한다. 그러나, 암에서 CREB의 역할은 알려진 바가 매우 적다. 인간의 에너지 항상성 유지와 에너지 스트레스 저항을 매개하는 CREB의 역할은 암세포가 주변 환경의 에너지 스트레스에 적응하고 살아남기 위해 스스로의 대사 프로그램을 재구성하는 현상과 유사하다. 따라서, CREB은 암에서도 스트레스 저항과 에너지 회복에 관련된 역할을 담당할 것으로 예상된다. 본 연구에서는 암세포에서 glucose 스트레스에 대한 CREB의 역할을 알아보고 glucose 결핍조건에서

CREB에 의해 발현이 조절되는 유전자에 대해 알아보고자 하였다.

방법: 여러가지 인간 암세포주 (A549, H1299, MDA-MB-231, MDA-MB-468, BT474, SKOV-3)를 이용하여 실험을 진행하였다. 대사성 스트레스를 흉내내기 위해 glucose 또는 소태아혈청을 제거한 배지를 이용하였다. CREB 특이적 역할을 확인하기 위해 siRNA를 이용한 transient transfection을 시행하였다. CREB의 대사적 특성을 확인하기 위해 정상 배양조건에서 세포증식 분석을 하였고 낮은 glucose와 소태아혈청을 제거한 배양조건에서 세포생존 분석을 시행하였다. 세포사 방식을 조사하기 PARP 분열을 Western blot으로 조사하였고 유세포 분석기를 이용한 세포사멸 분석을 시행하였다. Glucose가 제거된 상태에서 CREB 특이적인 유전자 발현을 확인하기 위해 microarray 실험과 gene set enrichment 분석을 시행하였고, 이 결과를 quantitative real-time-PCR을 이용하여 검증하였다.

결과: Glucose가 제거된 세포배양 조건에서 인산화된 CREB의 발현이 증가하였다. 정상 세포배양 조건에서 CREB-knockdown 세포의 세포증식이 대조군에 비해 유의하게 억제되었다 ($p < 0.001$). 대사성 스트레스 세포배양 조건에서 CREB-knockdown 세포의 생존이 대조군에 비해 유의하게 높았다 ($p < 0.001$). Glucose가 제거된 배양조건에서 PARP 분열이 CREB-knockdown에 의해 감소하였다. Glucose가 제거된 배양조건에서 사멸된 세포 수가 CREB-knockdown에 의해 감소하였다. Glucose가 제거된 배양조건에서 glycolysis/gluconeogenesis, oxidative

phosphorylation와 ATP synthesis 같은 에너지 대사와 관련된 gene sets의 발현이 CREB-knockdown에 따라 유의한 차이를 보였다 ($p < 0.00001$). Glucose가 제거된 배양조건에서 NDUFV1, ATP5G1와 MVD 유전자 같은 CREB target gene의 발현이 CREB-knockdown 세포에서 대조군에 비해 유의하게 감소하였다 ($p < 0.001$).

결론: CREB은 정상 배양조건에서 암세포 증식에 필수적이다. Glucose가 제거된 배양조건에서 CREB은 활성화되고 에너지대사와 관련된 gene sets의 발현과 관련되어 세포사멸을 매개한다. 이러한 결과는 암세포에서 glucose 스트레스 유무에 따른 CREB의 역동적인 역할을 제시하고 있다.

핵심되는 말 : cyclic AMP response element-binding protein, 암, glucose, 스트레스