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TITLE: Targeting Energy Metabolic Pathways as Therapeutic Intervention for Breast Cancer

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Targeting Energy Metabolic Pathways as Therapeutic Intervention for Breast Cancer

Cancer cells predominantly metabolize glucose by glycolysis to produce energy in order to meet their metabolic requirement, a phenomenon known as Warburg effect. Although Warburg effect is considered a peculiarity critical for survival and proliferation of cancer cells, the regulatory mechanisms behind this observation remain incompletely understood. We report here that eukaryotic elongation factor-2 kinase (eEF-2K), a negative regulator of protein synthesis, has a critical role in promoting glycolysis and tumor development and progression. Promotion of glycolysis by eEF-2K is mediated through the inhibitory effect of this kinase on translational elongation of protein phosphatase 2A-A (PP2A-A), a protein phosphatase that promotes the ubiquitin-proteasomal degradation of c-Myc. eEF-2K-mediated restriction of the synthesis of PP2A-A protein slows down the turnover of c-Myc, which activates the transcription of pyruvate kinase M2 isoform (PKM2), a key enzyme in the glycolytic pathway. In addition, depletion of eEF-2K reduced the ability of the transformed cells to proliferate and enhanced the sensitivity of tumor cells to chemotherapy both in vitro and in vivo. The results of this study not only provide the first connection between the regulation of protein synthesis and glycolysis activity, but also underscores the importance of eEF-2K in cancer, and the potential of this kinase as a novel target for prevention and treatment of this disease.
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INTRODUCTION

Cancer cells rely mainly on glycolysis to metabolize glucose for energy production even in the presence of sufficient oxygen, a phenomenon known as Warburg effect (1). This metabolic adaptation is believed to be critical for tumor cell growth and proliferation (2). Moreover, glycolytic cancer cells are often invasive and impervious to therapeutic intervention (3). Thus, altered energy metabolism is now appreciated as a hallmark of cancer and a promising target for cancer treatment. Despite the importance of Warburg effect in cancer development, progression and therapy, the precise molecular mechanisms regulating this peculiarity of cancer are still not fully elucidated.

eEF-2K (eukaryotic elongation factor-2 kinase, a.k.a. calcium/calmodulin-dependent protein kinase III), a member of the family of atypical \(\alpha\)-kinases, is an evolutionarily conserved regulator of protein synthesis. This kinase phosphorylates eEF-2 on Thr56 and inactivates this key elongation factor, blocking translation elongation and inhibiting global protein synthesis (4). A number of studies including our own have demonstrated that various stress such as growth factor derivation, nutrient deficiency, oxidative and chemical insults are potent stimulators of eEF-2K, and the activity of this kinase is required for survival of stressed cells (5-7). We also reported that eEF-2K is overexpressed in several types of cancer including breast cancer and glioma (8), and it plays a crucial role in regulating activity of autophagy and level of cellular ATP in tumor cells (5-7). As autophagy activity and ATP production are believed to be closely linked with rate of glycolysis, we queried whether eEF-2K has a role in this altered metabolic process that is appreciated as a peculiarity critical for tumor cell growth and proliferation. In this grant period, we found that expression of eEF-2K plays a promotive role in glycolysis and in tumor development and progression. Promotion of glycolysis by eEF-2K is mediated by its inhibitory effect on translation elongation of PP2A-A, a phosphatase that promotes the proteasomal degradation of c-Myc via dephosphorylating it. Restriction of synthesis of PP2A-A protein by eEF-2K slows down the turnover of c-Myc, which activates the transcription of pyruvate kinase M2 isoform (PKM2), a key
enzyme in the glycolytic pathway. The results of this study not only reveal a previously unappreciated role of eEF-2K in promoting glycolysis, but also present the eEF-2K/PP2A-A/c-Myc/PKM2 as a new pathway regulating this important metabolic process in cancer. These results also underscore the potential of eEF-2K as a novel target for prevention and treatment of cancer.

**BODY**

**TRAINING**

*Mentoring:*

According to my training plan, I meet with my primary mentor weekly, and the co-mentor every two months to discuss project goals. In the past three years, I have also had three meetings with my mentoring committee to discuss the research plan and progress.

In the grant period, I attended the following courses and program:

*Management and Career Independence:*

**Penn State Hershey Post-doctoral Society Workshop:**

*How to improve your CV & recommendation letters* (November 13, 2012)

*Negotiation workshop* (December 6, 2012)

*Professional development workshop on grant writing* (May 29, 2013)

*Professional Development Workshop on “Time Management”*(Oct 31, 2013)

*12th Annual Data & Dine*(Feb 25, 2014)

**Courses and Training Programs:**

*AACR Annual Meeting* (March 31–April 4, 2012)

*Annual Penn State Hershey Cancer Institute Symposium* (May 10, 2012)

*Annual Penn State College of Medicine Review Course for Clinical Oncologists* (July 20, 2012)

*Translational Research Symposium of Penn State Hershey Cancer Institute* (June 8, 2012)

*18th Annual Penn State Hershey Cancer Institute Symposium* (Oct 25, 2012)
RESEARCH

Task 1  To determine the importance of eEF-2K in energy metabolism of breast cancer cells.

In this Task, we have discovered:

1) Deficiency of eEF-2K caused a remarkable reduction of aerobic glycolysis in tumor cells

To understand the role of eEF-2K in altered energy metabolism in cancer, we determined the effects of this enzyme on aerobic glycolysis. Using siRNA we silenced the expression of eEF-2K in three human breast cancer cell lines (Fig. 1A), and then measured the glycolytic activity in these cells. We observed that the cells with knockdown of eEF-2K expression exhibited a decreased glucose consumption (Fig. 1B), as measured by flow cytometric analysis of the uptake of a fluorescent glucose analog (2-NBDG) (9). The glucose level was significantly higher in the conditioned medium of the cells with eEF-2K knockdown than that of the control cells (Fig. 1C), confirming that the glucose uptake was decreased in the eEF-2K-deficient cells. Consistently, the cells with knockdown of eEF-2K showed decreased levels of lactate (Fig. 1D), the final product of glycolysis, and ATP (Fig. 1E), as compared with the control cells. To further demonstrate the effect of eEF-2K on glycolysis, we inhibited the mitochondrial oxidative phosphorylation in these tumor cells using oligomycin, an inhibitor of ATP synthase or/and rotenone, an inhibitor of the electron transport chain, and then measured ATP production. Fig. 1F shows that a reduced production of ATP
remained to be observed when the eEF-2K knockdown cells were subjected to treatment with oligomycin or rotenone. These results further support a role for this kinase in promoting glycolysis. Similar effects of eEF-2K on uptake of glucose, and on productions of lactate and ATP, were observed in the Ras-transformed MEFseEF-2K(-/-) and MEFseEF-2K(+/-) (Fig. 1G).

2) **eEF-2K is required for the hypoxia-stimulated glycolysis and promoted survival of hypoxic tumor cells**

Hypoxic tumor microenvironment is believed to be an important factor contributing to the Warburg effect. Under hypoxia, tumor cells up-regulate glycolytic activity to meet the energy requirements for their survival and proliferation. We found that eEF-2K also had a role in boosting glycolysis when the breast cancer cells were subjected to hypoxia. Fig. 2A and Fig. 2B show that in comparison to the control, knockdown of eEF-2K expression suppressed the hypoxia-stimulated glycolysis in these tumor cells, as evidenced by decreased productions of lactate and ATP. Moreover, knockdown of eEF-2K expression led to an increased sensitivity of tumor cells to hypoxia, as indicated by a decrease in cellular viability (Fig. 2C) and an increase in apoptosis (Fig. 2D) in the hypoxic tumor cells with depletion of eEF-2K. These data suggest that the hypoxic tumor cells require eEF-2K to enhance glycolysis in support of their survival.

**Task 2 To determine the role of eEF-2K in tumor cell growth and proliferation.**

In light of the promotive role of eEF-2K in glycolysis, and glycolysis is believed to favor survival and growth of tumor cells, we hypothesized that expression of eEF-2K may contribute to tumorigenesis. To test this hypothesis, we transformed mouse embryonic fibroblast (MEF) cells with wild-type (+/+) or homozygous disruption of eEF-2K (-/-) by expressing an activated form of H-Ras (H-RasV12) (Fig. 3A). As shown in Fig. 3B, the H-RasV12-MEFs eEF-2K+/+ proliferated significantly slower than the H-RasV12-MEFs eEF-2K+/- H-RasV12-MEFs eEF-2K-/- also exhibited a significant decreased ability of anchorage-independent growth in soft agar colony formation assay (Fig. 3C). In nude mice, the H-RasV12-MEFs eEF-2K-/- formed smaller tumors than the H-RasV12-MEFs eEF-
These results suggest that eEF-2K confers a growth advantage to the transformed cells. Similarly, the human breast cancer cells with stable knockdown of eEF-2K expression formed fewer colonies (Fig. 3E), showed a decreased proliferation rate in vitro (Fig. 3F) and in vivo (Fig. 3G), and formed significantly smaller tumors in mice (Fig. 3H), as compared to the control cells without knockdown of the kinase.

**Task 3  To determine how eEF-2K promotes cell metabolism in breast cancer cells.**

*In this Task, we have discovered:*

1) **Promotion of glycolysis by eEF-2K is mediated through PKM2.**

To explore the molecular mechanisms by which eEF-2K promotes glycolysis, we examined the effects of eEF-2K expression on several key players in the glycolytic pathway. In these experiments, we found that PKM2 and c-Myc were down-regulated in the tumor cells with knockdown of eEF-2K (Fig. 4A). The activity of PKM2 and the amount of pyruvate, the product of the reaction catalyzed by PKM2, were also significantly decreased in the eEF-2K knockdown cells (Fig. 4B and Fig. 4C). Furthermore, knockdown of eEF-2K expression led to a reduction in PKM2 mRNA expression, suggesting that eEF-2K may affect PKM2 expression at transcription level (Fig. 4D). Down-regulations of PKM2 mRNA and the protein in the cells deficient in eEF-2K were also observed in the Ras-transformed MEFs with knockout of eEF-2K gene (Fig. 4E). To validate the effects of eEF-2K on PKM2 in these cells, we also overexpressed this kinase using an expression plasmid, and then examined the levels of PKM2 mRNA and the protein. Fig. 4F and Fig. 4G show that both of the protein and the mRNA of PKM2 were increased in the cells transfected with the eEF-2K expression plasmid. Furthermore, the reductions of glucose consumption and lactate production found in the eEF-2K knockdown cells could be reversed by PKM2 overexpression (Fig. 4H and Fig. 4I). Suppression of the expression and activity of PKM2 and inhibition of pyruvate production by knockdown of eEF-2K expression were
recapitulated in the hypoxic tumor cells (Fig. 4J, Fig. 4K and Fig. 4L). These results suggest that PKM2 is a mediator of the effect of eEF-2K on glycolysis.

2) eEF-2K activates PKM2 through stabilizing c-Myc protein.

We next sought to understand how eEF-2K exerts its regulatory effects on PKM2. As we also found that c-Myc, a transcription factor reported to enhance the generation of PKM2 mRNA by splicing through transcriptionally activating hnRNP proteins (10), was down-regulated in the cells subjected silencing of eEF-2K expression (Fig. 4A), we therefore asked whether there was a causal link between the down-regulation of c-Myc and PKM2 in the eEF-2K knockdown cells. To test this hypothesis, using c-Myc-targeted siRNA and c-Myc expression plasmid, we first showed that the expression level of c-Myc indeed affected the expression of PKM2 mRNA in MDA-MB-231 and MCF-7 cells, as determined by real-time quantitative RT-PCR (Fig. 5A and Fig. 5B). Our next question then is how eEF-2K affects c-Myc expression. Although we observed that c-Myc protein was down-regulated in the eEF-2K-deficient cells (Fig. 4A), the amount of c-Myc mRNA was identical in cells with or without silencing of eEF-2K expression (Fig. 5C), suggesting that this kinase does not affect c-Myc transcription. However, pulse-chase experiments demonstrated that knockdown of eEF-2K enhanced the turnover of the c-Myc protein (Fig. 5D). As the degradation of c-Myc protein is mediated by the ubiquitin-proteasome pathway (11), we further demonstrated that MG132, a proteasome inhibitor, rescued the down-regulation of c-Myc in the cells depleted of eEF-2K (Fig. 5E). These results suggest that the function of eEF-2K may be involved in stabilizing the c-Myc protein.

Next, we queried about the molecular mechanism by which eEF-2K affects the stability of c-Myc protein. The known function of eEF-2K is to phosphorylate eEF-2 at Thr56, thereby inactivating this elongation factor and negatively regulating global protein synthesis. Here, we sought to determine whether depletion of eEF-2K had any effect on expression of PP2A, a phosphatase known to promote the proteasomal degradation of c-Myc via dephosphorylating it at Ser62 (12). We found that in the eEF-2K knockdown cells,
the amount of PP2A subunit A protein was increased, as compared with the control cells (Fig. 6A). PP2A subunit C and PP1 protein levels were similar in both of the control and the eEF-2K knockdown cells (Fig. 6A). We next wanted to determine whether the elevation of PP2A-A level in the eEF-2K knockdown cells resulted from increased synthesis of the protein. Because eEF-2K slows down protein synthesis through an inhibitory phosphorylation on eEF-2, which terminates peptide elongation, we first tested the dependency of PP2A-A expression on eEF-2. Fig. 6B demonstrates that when eEF-2 expression knocked down, the expression of PP2A-A protein was dramatically inhibited. To more directly demonstrate the role of eEF-2 in regulation of PP2A-A protein translation, we measured the binding of PP2A-A mRNA to eEF2 in the immunoprecipitates of eEF-2. In these experiments, cell extracts of MDA-MB-231 were incubated with an eEF2 antibody or an isotype control IgG, and the presence of PP2A-A mRNA in the immunoprecipitates was analyzed using quantitative real-time PCR. Fig. 6C shows that a high level of PP2A-A mRNA was detected in the anti-eEF2 immunoprecipitates, indicating that the PP2A-A mRNA selectively associates with eEF2. Importantly, silencing of eEF-2K expression significantly enhanced the association of PP2A-A mRNA with eEF2 (Fig. 6D), suggesting that the translation elongation of PP2A-A is promoted by de-repression of eEF-2. Collectively, these data suggest that eEF-2K is critical for control of PP2A-A translation elongation through regulating the activity of eEF-2.

**Task 4** To determine whether targeting eEF-2K can enhance the antitumor efficacy of chemotherapeutic drugs.

It has been known that inhibiting glycolysis may sensitize cancer cells to chemotherapy. Here we tested the effects of depleting eEF-2K on sensitivity of tumor cells to doxorubicin and paclitaxel, two chemotherapeutic drugs commonly used in treatment of breast cancer. We found that the breast cancer cells with knockdown of eEF-2K were more sensitive to doxorubicin and paclitaxel than the cells without knockdown of eEF-2K (Fig. 7A and Fig. 7B). Moreover, suppression of eEF-2K expression by RNAi could also significantly increase the
cytotoxicity of paclitaxel in the multidrug-resistant MCF-7/ADR cells (Fig. 7C). The sensitizing effect of targeting eEF-2K on chemotherapy was also observed in mice bearing MDA-MB-231 tumors treated with paclitaxel (Fig. 7D). These results suggest that suppression of eEF-2K to reinforce the antitumor efficacy of chemotherapeutic drugs may be explored as a new therapeutic strategy for more effective treatment of malignant tumors.

**KEY RESEARCH ACCOMPLISHMENTS**

- We found that eukaryotic elongation factor-2 kinase (eEF-2K), a negative regulator of protein synthesis, promotes glycolysis in breast cancer.
- We showed that depletion of eEF-2K decreased the ability of transformed cells and breast cancer cells to proliferate.
- We demonstrated that eEF-2K/PP2A/c-Myc/PKM2 signaling cascade is critical for eEF-2K-mediated aerobic and tumorigenesis.
- We showed that suppression of eEF-2K enhanced the sensitivity of tumor cells to chemotherapy both in vitro and in vivo.
- We provide the first connection between the regulation of protein synthesis and glycolysis activity, and underscores the potential of this kinase as a novel target for prevention and treatment of this disease.

**REPORTABLE OUTCOMES**

Abstract


**Cheng Y**, Ren XC, Zhang L, Yang JM: Identification of RSK2 as a novel regulator

**Published Papers**


**Award**

Penn State Hershey Annual Outstanding Postdoctoral Scholars and Fellows Award (Feb 21, 2012)

**CONCLUSIONS**

The results of this study not only reveal a previously unappreciated role of eEF-2K in promoting glycolysis, but also present the eEF-2K/PP2A-A/c-Myc/PKM2 as a new pathway regulating this important metabolic process in cancer. These results also underscore the potential of eEF-2K as a novel target for prevention and treatment of cancer.

**REFERENCES**


APPENDIX
Figure 1. Inhibition of eEF-2K reduces glycolysis in human breast cancer cell lines and the transformed MEFs. MCF-7, MDA-MB-231 and MDA-MB-468 cells were transfected with a non-targeting or eEF-2K siRNA. (A) eEF-2K protein expression was examined by western blot. Tubulin was used as a loading control. (B) The glucose consumption was determined by measuring uptake of 2-NBDG using flow cytometry. (C) The concentration of glucose from extracellular medium was measured by Amped® Red glucose/glucose oxidase assay kit. Results are normalized to control. (D) The concentration of L-lactate released to the cell culture medium was measured by L-lactate assay kit. (E) Cellular ATP level was measured by CellTiter-Glo® luminescent assay. Results are normalized to control. (F) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA, followed by oligomycin or rotenone treatment. Cellular ATP level was measured by CellTiter-Glo® luminescent assay. Results shown in B, C, D, E and F were normalized to the control; each bar represents the mean ± SE of three identical experiments. ** *p* <0.01. (G) Glucose level in the conditioned medium, level of L-lactate released to the cell culture medium and cellular ATP level, were measured in the Ras-transformed MEFs eEF-2K(-/-) and MEFs eEF-2K(+/-). Results shown were normalized to the control. Each bar represents the mean ± SE of three identical experiments. **p< 0.01.
Figure 2. Silencing of eEF-2K expression blunts the hypoxia-stimulated glycolysis and reduces survival of hypoxic tumor cells. MDA-MB-231 or MCF-7 cells were transfected with a non-targeting RNA (control) or an eEF-2K siRNA, followed by incubation in a hypoxia chamber for 24 h. (A) At the end of incubation, concentrations of L-lactate released to the cell culture medium were measured. (B) At 0, 12 and 24 h following hypoxia treatment, cellular ATP levels were assayed. (C and D) At 0, 24 and 48 h following hypoxia treatment, cellular viability (C) and apoptosis (D) were determined. Each bar represents the mean ± SE of three identical experiments. *p < 0.05, **p < 0.01.
Figure 3. eEF-2K promotes cell proliferation in Ras-transformed epithelial cells and human breast cancer cells. (A) The expressions of p-eEF-2 and Ras were determined by western blot in eEF-2K+/+ and eEF-2K−/− MEFs expressing empty vector or H-RasV12. Tubulin was used as a loading control. (B) The growth curve of H-RasV12- MEFs eEF-2K−/− and H-RasV12-MEFs eEF-2K+/+. (C) Anchorage-independent cell growth of H-RasV12-transformed eEF-2K WT and KO MEFs. (D) The tumor formation of H-RasV12-transformed eEF-2K WT and KO MEFs was examined in vivo. (E) Colony formation of MDA-MB-231 cells stably expressing control or eEF-2K shRNA. (F) The growth curves of MDA-MB-231 cells stably expressing control or eEF-2K shRNA. (G) Immunohistochemical staining for Ki67 in the tumor xenografts. (H) The tumor formation of MDA-MB-231 cells stably expressing control or eEF-2K shRNA was examined in vivo.
Figure 4. eEF-2K is a positive regulator of PKM2. MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA. (A) eEF-2K, PKM2 and c-Myc protein expressions, (B) The activity of PKM2, (C) The level of pyruvate; and (D) The expression of PKM2 mRNA, were measured. (E) Expression of PKM2 mRNA in the HRas-MEFs eEF-2K+/+ and HRas-MEFs eEF-2K−/− was measured by qRT-PCR, as described in “Materials and Methods”. Expression of c-Myc, PKM2, and PP2A-A protein were determined by western blot. Tubulin was used as a loading control. (F and G) MDA-MB-231 or MCF-7 cells were transfected with a control vector or eEF-2K expression plasmid. Expression of eEF-2K and PKM2 protein (F) and PKM2 mRNA (G) were measured. MDA-MB-231 or MCF-7 cells were transfected with a non-targeting RNA (control) or an eEF-2K siRNA, followed by transfection with a PKM2 expression plasmid. The concentrations of glucose in the conditioned medium (H) and the amounts of lactate released to the cell culture medium (I) were measured. Results shown were normalized to the control; each bar represents the mean ± SE of three identical experiments. *p < 0.05, **p < 0.01. MDA-MB-231 or MCF-7 cells were transfected with a non-targeting RNA (control) or an eEF-2K siRNA, followed by hypoxia treatment. At the end of treatment, PKM2 mRNA (J), PKM2 activity (K), and pyruvate level (L) were measured. Each bar represents the mean ± SE of three identical experiments. *p < 0.05, **p < 0.01.
Figure 5. c-Myc regulates the expression of PKM2 mRNA, inhibition of eEF-2K promotes the degradation of c-Myc. (A) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or c-Myc siRNA. The expression of PKM2 mRNA was measured by qRT-PCR. (B) MDA-MB-231 and MCF-7 cells were transfected with a control vector or c-Myc plasmid. The expression of PKM2 mRNA was measured by qRT-PCR. (C) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA. The expression of c-Myc mRNA was measured by qRT-PCR. (D) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA, followed by CHX treatment. c-Myc protein expression was examined by Western blot. Tubulin was used as a loading control. (E) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA, followed by MG132 treatment. eEF-2K and c-Myc protein expressions were examined by Western blot. Tubulin was used as a loading control.

![Figure 5](image)

Figure 6. eEF-2K controls PP2A-A translation through phosphorylation of eEF-2. (A) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA. eEF-2K, PP2A-A, PP2A-C and PP1 protein expressions were examined by Western blot. Tubulin was used as a loading control. (B) MDA-MB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2 siRNA. eEF-2, PP2A-A, PP2A-C and PP1 protein expressions were examined by Western blot. Tubulin was used as a loading control. (C) PP2A-A mRNA was detected by qRT-PCR in immunoprecipitates obtained with anti-eEF2. mRNA amounts are expressed relative to the amount detected in control IgG immunoprecipitates. (D) MDA-MB-231 cells were transfected with a non-targeting or eEF-2K siRNA, and PP2A-A mRNA was detected by qRT-PCR in immunoprecipitates obtained with anti-eEF2.

![Figure 6](image)
Figure 7. Knockdown of eEF-2K expression enhances sensitivity of breast cancer cells to paclitaxel and doxorubicin. (A and B) MDA-MB-231 or MCF-7 cells were transfected with a non-targeting RNA or an eEF-2K siRNA, followed by treatment with doxorubicin (A) or paclitaxel (B) for 72 h. (C) Multidrug resistant MCF-7/ADR cells were transfected with a non-targeting RNA or an eEF-2K siRNA, followed by paclitaxel treatment for 72 h. Cell viability was measured by MTT assay. Each bar represents the mean ± SE of three experiments. *, p<0.05; **, p < 0.01, t-test. (D) Nude mice were inoculated s.c. with MDA-MB-231 cells with or without silencing of eEF-2K expression, and then the mice bearing tumors were treated with paclitaxel. The tumor sizes were measured on days as indicated.