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Regulation of G_1 Arrest and Apoptosis in Hypoxia by PERK and GCN2-Mediated eIF2α Phosphorylation[1,2]

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Abstract

Hypoxia is a common microenvironment in solid tumors and is correlated with tumor progression by regulating cancer cell survival. Recent studies suggest that activation of double-stranded RNA-dependent protein kinase–like endoplasmic reticulum–related kinase (PERK) and phosphorylation of α subunit of eIF2 (eIF2α) confer cell adaptation to hypoxic stress. However, eIF2α is still phosphorylated at a lowered level in PERK knockout cells under hypoxic conditions. The mechanism for eIF2α kinase(s) (EIF2AK)–increased cell survival is not clear. In this report, we provide evidence that another EIF2AK, the amino acid starvation–dependent general control of amino acid biosynthesis kinase (GCN2), is also involved in hypoxia-induced eIF2α phosphorylation. We demonstrate that both GCN2 and PERK mediate the cell adaptation to hypoxic stress. High levels of eIF2α phosphorylation lead to G_1 arrest and protect cells from hypoxia-induced apoptosis. Reduced phosphorylation of eIF2α by knocking out either PERK or GCN2 suppresses hypoxia-induced G_1 arrest and promotes apoptosis in accompany with activation of p53 signal cascade. However, totally abolishing phosphorylation of eIF2α inhibits G_1 arrest without promoting apoptosis. On the basis of our results, we propose that the levels of eIF2α phosphorylation serve as a "switch" in regulation of G_1 arrest or apoptosis under hypoxic conditions.

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Introduction

Cells respond to external stimuli by rapid changes in their translational capacity. Stress, such as growth factor depletion, heat shock, and virus infection, rapidly inhibits protein synthesis through phosphorylation of the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) [1,2]. Four kinases, the double-stranded RNA-dependent protein kinase (PKR), the hemin-regulated inhibitor (HRI), the amino acid starvation–dependent general control of amino acid biosynthesis kinase (GCN2), and the PKR-like endoplasmic reticulum–related kinase (PERK), have been identified to phosphorylate eIF2α and reduce translation initiation in response to stress [2]. Recently, PERK-mediated phosphorylation of eIF2α has been shown to suppress protein synthesis and cell growth on hypoxia [3–6]. *In vivo* studies show that hypoxia-induced activation of PERK inhibits protein synthesis, decreases cell growth, and promotes tumor adaptation [2–6].

Adaptation to hypoxia could also be regulated by hypoxia-inducible factor 1 (HIF-1), which associates with tumor progression and resistance to radiotherapy and chemotherapy [2,7,8]. HIF-1 is a key mediator in hypoxia [9] and regulates the expressions of more than 70 genes [10] that facilitate metabolic adaptation, cell proliferation, cell cycle arrest, apoptosis, angiogenesis, angioinvasion, and metastasis [11,12].

Abbreviations: eIF2α, the α subunit of the eukaryotic initiation factor-2; EIF2AK, eIF2α kinase; GCN2, amino acid starvation–dependent general control of amino acid biosynthesis kinase; HIF-1α, hypoxia-inducible factor 1 α; HRI, hemin-regulated inhibitor; Mdm2, murine double minute 2; MEF, mouse embryonic fibroblast; PERK, PKR-like endoplasmic reticulum–related kinase; PKR, double-stranded RNA-dependent protein kinase

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HIF-1 is a heterodimer composed of α and β subunits. The expression of HIF-1β is constitutive, whereas the level of HIF-1α is highly regulated by oxygen levels [9]. Whereas HIF-1α undergoes rapid degradation and is maintained at basal levels in normoxia, it accumulates through protein stabilization and/or increased expression under hypoxia [13,14]. HIF-1α coordinates with p53, murine double minute 2 (Mdm2), and $p21^{WAF1}$ in the regulation of cell cycle arrest and apoptosis under hypoxic conditions. However, the regulatory mechanism is controversial [15–17]. There are reports indicating that HIF-1α forms a complex with p53 and stabilizes p53 [18], which promotes cell cycle arrest mediated by $p21^{WAF1}$ and induces apoptosis [19]. Mdm2 negatively regulates p53 by promoting p53 degradation [20]. Other reports suggest that HIF-1α does not directly associate with p53, but with Mdm2 [17,21], which upregulates HIF-1α levels [17,22,23]. The mechanism for hypoxia-mediated cell cycle arrest and apoptosis remains unclear [24–26]. In this report, we provide pieces of evidence that translation initiation plays a critical role in regulation of hypoxia-induced signaling circuit. Under hypoxic conditions, PERK and GCN2 are activated and coordinately phosphorylate eIF2α. Depending on the levels of eIF2α phosphorylation, the expression and activity of HIF-1α, p53, Mdm2, and $p21^{WAF1}$ are altered under hypoxic conditions. Our findings not only significantly advance the understanding of the mechanism for hypoxia/eIF2α phosphorylation–mediated G_1 arrest and apoptosis signaling circuit but also lead to the discovery of a potential target for antitumor therapies.

Materials and Methods

Cell Culture and Hypoxic Treatments

Mouse embryonic fibroblast (MEF) wild type (MEF^{WT}), MEF PERK knockout ($MEF^{PERK-/-}$), MEF GCN2 knockout ($MEF^{GCN2-/-}$), and MEF S51A mutant ($MEF^{A/A}$), in which the wild type eIF2α was replaced with a nonphosphorylatable S51A mutated eIF2α, were kindly provided by Dr. RJ Kaufman (University of Michigan Medical School, Ann Arbor, MI). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Cellgro, Manassas, VA) supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum (Cellgro) at 37°C with 5% CO_2. GasPak EZ Anaerobe Pouch System (BD Biosciences, VWR, S. Plainfield, NJ) was used to reduce the oxygen levels to less than 1% (mean, 0.7%) in 90 minutes.

Western Blot Analysis

The cells were washed with cold phosphate-buffered saline (PBS) twice and lysed in buffer with 50 mM Tris-HCl, 150 mM NaCl, 0.05% EDTA, 0.5% IGEPAL CA-630, and a cocktail of protease inhibitors (Roche, Indianapolis, IN). An equal amount of total proteins was separated by SDS-PAGE. Protein was electroblotted onto Immobilon-P membrane (Millipore, Temecula, CA), which was then blocked with 5% milk in PBST (PBS with 0.1% Tween-20). The interested proteins were probed with the corresponding antibodies and visualized by LumiGLO reagent and peroxide (Cell Signaling, Danvers, MA). Anti-eIF2α, anti–phosphorylated eIF2α (Ser 51), and anti–β-actin were purchased from Sigma (St. Louis, MO). Anti-Mdm2 (N-20), anti-p53 (Bp53), anti–HIF-1α, and secondary horseradish peroxidase–linked antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Viability Assay

The cells (1×10^5) were seeded in 12-well plates and were then exposed to normoxia or hypoxia. The viability was measured at different time points by the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's protocol. After removing the medium, 75 μl of CellTiter-Glo reagent was diluted once with water and added into each well. The cells were incubated with reagent at room temperature on a shaker for 5 minutes and then incubation was continued for 10 minutes. Luminescence of each sample (100 μl) was measured by a Lumat LB9507 luminometer (Berthold Technologies, Oak Ridge, TN).

Cell Cycle Analysis

The cells (2×10^6) were seeded in 100-mm tissue culture plates and exposed to normoxia or hypoxia. The cells were harvested and washed twice with cold PBS. The cells were suspended in 200 μl of PBS and fixed in 4 ml of cold 70% ethanol at −20°C overnight. The cells were centrifuged at 4°C for 10 minutes. The cell pellets were stained by a propidium iodide mixture (100 μg/ml RNase and 50 μg/ml propidium iodide) at 37°C for 30 minutes. The amount of apoptotic cells in a total of 1×10^4 cells was determined by a flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by ModFit LT (Verity Software House, Topsham, ME).

Apoptosis Assay

The cells (2×10^5) were seeded in 12-well plates and exposed to normoxia or hypoxia for 2 days. The cells were analyzed by a Cell Death Detection ELISA kit (Roche Diagnostics), which measures cleaved histone and DNA complex. The assay was performed according to the manufacturer's manual. The absorbance at 405/590 nm was measured by a SPECTRA Max M2 multichannel fluorescence plate reader (Molecular Devices, Sunnyvale, CA).

Luciferase Assay

Cells were seeded in 24-well plates and cotransfected with an inducible luciferase expression vector and a Renilla luciferase expression vector (Panomics, Fremont, CA). At 24 hours after transfection, the cells were exposed to normoxia or hypoxia. The luciferase activities were measured by a Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's manual. Luciferase and Renilla luciferase activities were measured by a Lumat LB 9507 luminometer (Berthold Technologies). The Renilla luciferase activity was used to normalize the transfection efficiency.

Glucose Uptake Assay

The cells were washed with serum-free DMEM twice and then cultured under hypoxia with serum-free medium for 12 hours. The cells were washed with Kreb's Ringer phosphate (CRP) buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4) twice and then incubated with 450 μl of KRP buffer for 30 minutes. Fifty microliters of KRP supplemented with 1 μCi/ml [^{3}H] 2-deoxy-D-glucose and 1 mM glucose was added to each sample and incubated for 30 minutes at 4°C. The buffer was removed, and the cells were washed twice with cold PBS. Cells were lysed with 350 μl of 0.2 M NaOH. Radioactivity was determined by LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

Clonogenic Assay

The cells (5×10^3) were seeded in six-well plates and cultured in 95% air and 5% CO_2 at 37°C for 6 days. The cells were then washed with PBS twice and fixed by cold methanol for 10 minutes at −20°C. The fixed cells were stained by 1% crystal violet in 25% methanol for 10 minutes

at room temperature. The cells were finally rinsed with distilled water, and the colonies with a size more than 0.5 mm were counted.

Statistical Analysis

Student's *t* test was used to analyze the significance of data. $P < .05$ was considered significant.

Results

Hypoxia-Induced Cell Death Is Protected by PERK and GCN2 in an eIF2α Phosphorylation-Independent Manner

PERK has been suggested to mediate the hypoxia-induced phosphorylation of eIF2α and increase tolerance of tumor cells to hypoxic stress [3,4,26]. However, whereas the thapsigargin-induced eIF2α phosphorylation is totally inhibited in $MEF^{PERK-/-}$ cells, the hypoxia-induced phosphorylation is only partially inhibited in the same cells [26]. The results suggested that other eIF2α kinase (EIF2AK) besides PERK might also be involved in the hypoxia-induced eIF2α phosphorylation. Our recent study demonstrated that GCN2 coordinates with PERK in regulation of UVB-induced phosphorylation of eIF2α [27]. To determine whether GCN2 is also involved in hypoxia-induced phosphorylation of eIF2α, we analyzed time-dependent phosphorylation of eIF2α in MEF^{WT}, $MEF^{PERK-/-}$, $MEF^{GCN2-/-}$, and $MEF^{A/A}$ cells after modest hypoxia treatment. As expected, whereas eIF2α was not phosphorylated in $MEF^{A/A}$ cells, an increased phosphorylation of eIF2α was detected at 2 hours after treatment in the other cell lines.

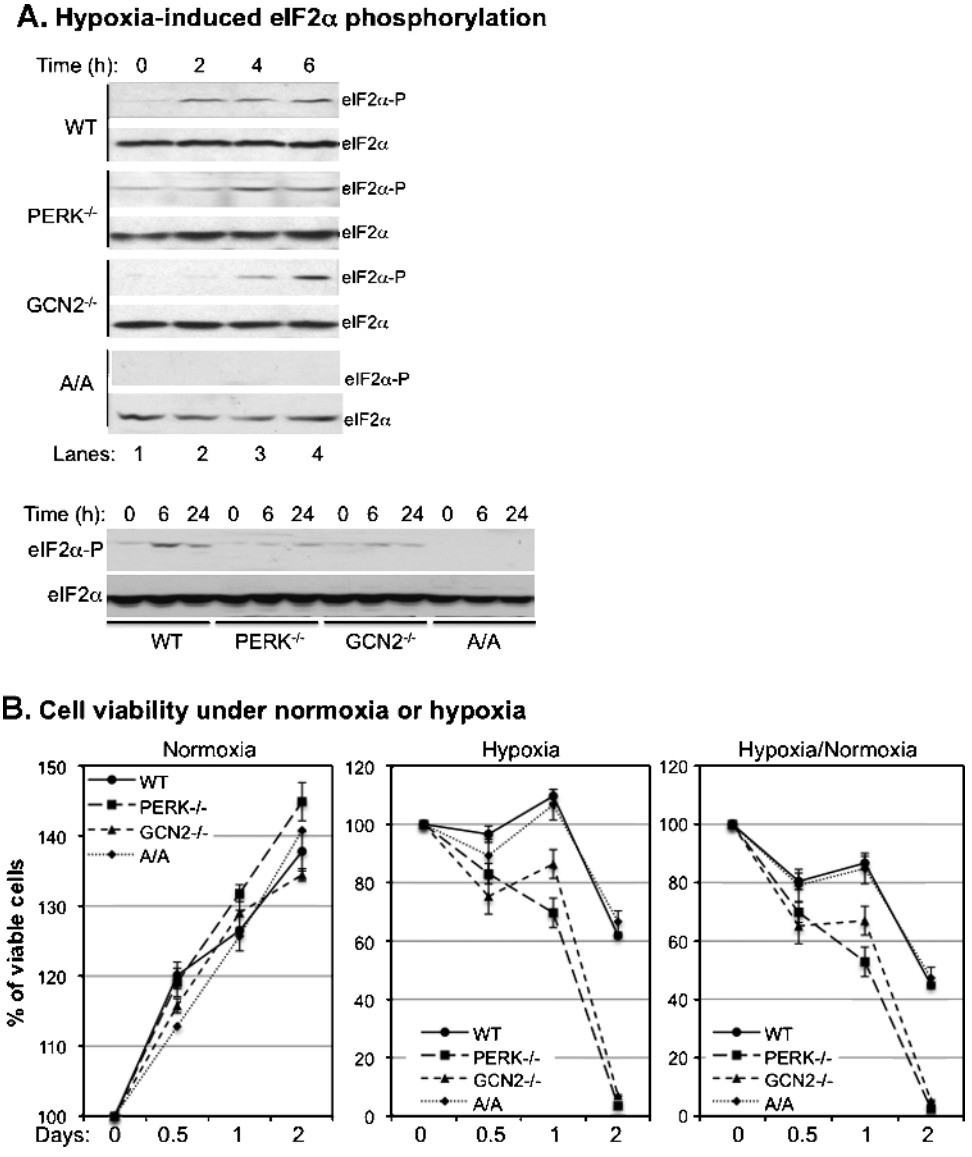

Figure 1. Both PERK and GCN2 mediate hypoxia-induced eIF2α phosphorylation and cell death. MEF^{WT}, $MEF^{PERK-/-}$, $MEF^{GCN2-/-}$, and $MEF^{A/A}$ cells were used in the experiments as indicated. (A) The cells were exposed to hypoxia for the indicated time points before immunoblot analysis with phosphorylated eIF2α (Ser 51) and total eIF2α antibodies. (B) The cells were exposed to normoxia or hypoxia for the indicated time points and then viability assays were performed. The bars represent the means of three independent experiments. (C) The cells were exposed to hypoxia for 12 hours with serum-free DMEM and were then fed with glucose supplemented with [^{3}H] 2-deoxy-D-glucose. Total protein was collected, and the radioactivity was measured by liquid scintillation counter. The bars represent the means of three independent experiments. *$P < .05$ mutant *versus* wild type. (D) The cells were exposed to normoxia or hypoxia for indicated time points and photographed using microscopy equipped with a Nikon digital camera (Nikon, West Chester, OH).

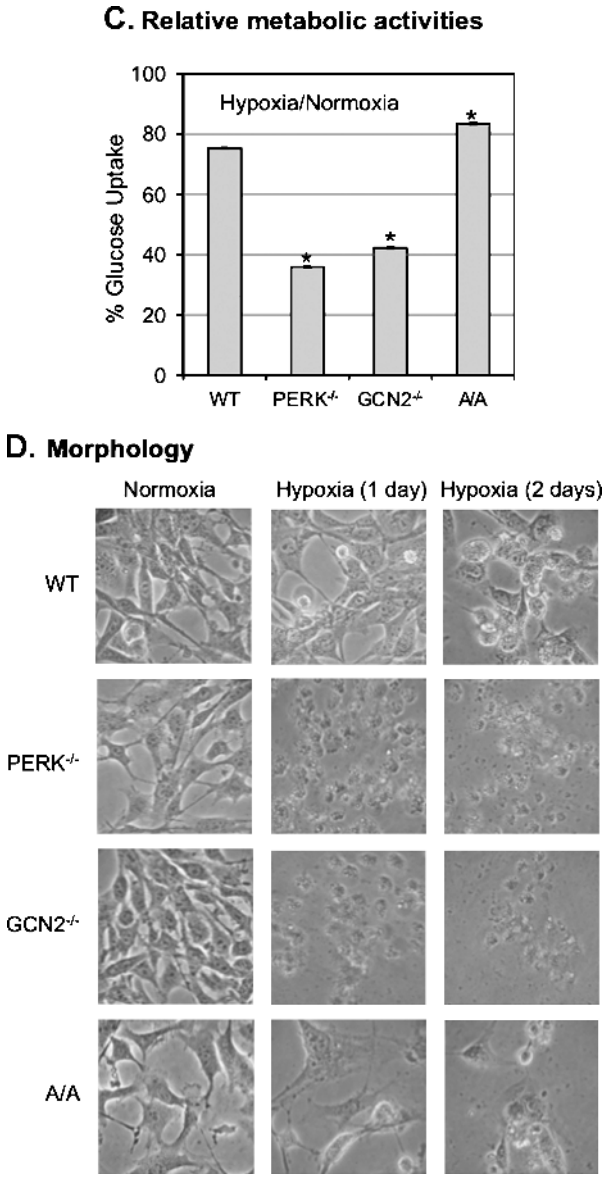

Figure 1. (*continued*).

Compared with MEF^{WT} cells, the hypoxia-induced phosphorylation of eIF2α was delayed and reduced in MEF$^{PERK-/-}$ cells (Figure 1*A*), which agreed with previous reports [26,28]. Moreover, our data also showed that the phosphorylation of eIF2α was similarly delayed and reduced in MEF$^{GCN2-/-}$ cells (Figure 1*A*). These results demonstrate that both PERK and GCN2 are involved in the hypoxia-induced phosphorylation of eIF2α.

It has been found that hypoxia-induced PERK activation protects cells from death by increasing eIF2α phosphorylation [3]. To determine whether GCN2 can also protect cells from hypoxia-induced death, we analyzed viabilities of MEF^{WT}, MEF$^{PERK-/-}$, MEF$^{GCN2-/-}$, and MEF$^{A/A}$ cells under normoxic or hypoxic conditions. Our data showed that, under normoxic conditions, the growth rates of MEF^{WT}, MEF$^{PERK-/-}$, MEF$^{GCN2-/-}$, and MEF$^{A/A}$ were within 10% differences (Figure 1*B*, *Normoxia*), but under hypoxic conditions, viabilities of MEF$^{GCN2-/-}$ and MEF$^{PERK-/-}$ cells decreased more rapidly than MEF^{WT} and MEF$^{A/A}$ cells (Figure 1*B*, *Hypoxia*). After correcting the survival rates in hypoxia with the growth rates in normoxia, our data demonstrated that the survival rates for MEF$^{GCN2-/-}$ and MEF$^{PERK-/-}$ cells were decreased in 48 hours to 2.4 ± 0.1% and 5.1 ± 0.3%, respectively (Figure 1*B*, *Hypoxia/Normoxia*). Surprisingly, the survival rate of MEF$^{A/A}$ cells was 47.3 ± 3.2%, which was similar to the 44.9 ± 2.1% survival rate of MEF^{WT} cells (Figure 1*B*, *Hypoxia/Normoxia*).

To further confirm the above results, we analyzed cell viabilities using metabolic and morphologic analyses. Glucose uptake assay was used to measure the metabolic activities of the cells in hypoxia. Our data showed that whereas the metabolic activities of MEF^{WT} and MEF$^{A/A}$ cells were maintained at 75.4 ± 0.3% and 83.5 ± 0.4%, respectively, the activities in MEF$^{GCN2-/-}$ and MEF$^{PERK-/-}$ cells were decreased to 36.0 ± 0.2% and 42.3 ± 0.2%, respectively (Figure 1*C*). In addition, the morphologic analysis also showed that the MEF$^{GCN2-/-}$ and MEF$^{PERK-/-}$ cells were more sensitive to hypoxia than MEF^{WT} and MEF$^{A/A}$ cells (Figure 1*D*). These results confirmed that the hypoxia-induced cell death depended on but not linearly related to the levels of eIF2α phosphorylation.

To determine whether PERK or GCN2 influences cell survival in hypoxia independently of eIF2α, we tried to knockout PERK or GCN2 in the MEF$^{A/A}$ cells using a siRNA method. Our results showed that when PERK or GCN2 siRNA reduced the viability of MEF^{WT} cells under hypoxia, they did not affect the viability of MEF$^{A/A}$ cells (Figure W1*A*). The preliminary result suggests that PERK- and GCN2-mediated hypoxia-induced cell death is eIF2α-dependent. However, our data are not conclusive because the transfection efficiency is less than 20% based on the control FITC-labeled scramble siRNA (Figure W1*B*).

Hypoxia-Induced Cell Cycle Arrest Is Dependent on PERK and GCN2 by Regulating HIF-1α and p21^{WAF1}

Both PERK and GCN2 contribute to cell cycle arrest on endoplasmic reticulum stress [29]. To determine whether both PERK and GCN2 play a role in the regulation of cell cycle arrest in hypoxia, we performed cell cycle analysis in MEF^{WT}, MEF$^{GCN2-/-}$, MEF$^{PERK-/-}$, and MEF$^{A/A}$ cells. Our data showed that hypoxia induced G_1 arrest in MEF^{WT} cells but not in MEF$^{PERK-/-}$, MEF$^{GCN2-/-}$, or MEF$^{A/A}$ cells (Figure 2*A*). These data demonstrate that the activation of PERK/GCN2 and the phosphorylation of eIF2α are essential for hypoxia-induced cell cycle arrest.

To assess the molecular mechanism for PERK/GCN2–mediated eIF2α phosphorylation in regulation of cell cycle arrest, we analyzed the expressions of HIF-1α and p21^{WAF1}, the key cell cycle regulators in hypoxia [25,30]. Our data showed that HIF-1α expression was induced in MEF^{WT} (Figure 2*B*). The hypoxia-inducibility of HIF-1α expression was eliminated or reduced in MEF$^{PERK-/-}$ and MEF$^{GCN2-/-}$ cells (Figure 2*B*). HIF-1α was not detected in MEF$^{A/A}$ cells in either normoxia or hypoxia (Figure 2*B*). In contrast to HIF-1α, the maximal inducibility of p21^{WAF1} in hypoxia was achieved in MEF^{WT} cells (Figure 2*B*). The inducibility, but not the expression levels of p21^{WAF1}, was correlated to the hypoxia-induced cell cycle arrest. These results suggest that the hypoxia-induced cell cycle arrest is mediated by the inductions of HIF-1α and p21^{WAF1}, which is regulated by PERK/GCN2–mediated eIF2α phosphorylation.

PERK and GCN2 Protect Cells from Hypoxia-Induced Apoptosis

To determine whether GCN2, like PERK, protects cells from hypoxia-induced apoptosis, we assessed the roles of PERK/GCN2–mediated eIF2α phosphorylation in regulation of apoptosis under hypoxia. MEF^{WT}, MEF$^{PERK-/-}$, MEF$^{GCN2-/-}$, and MEF$^{A/A}$ cells were exposed

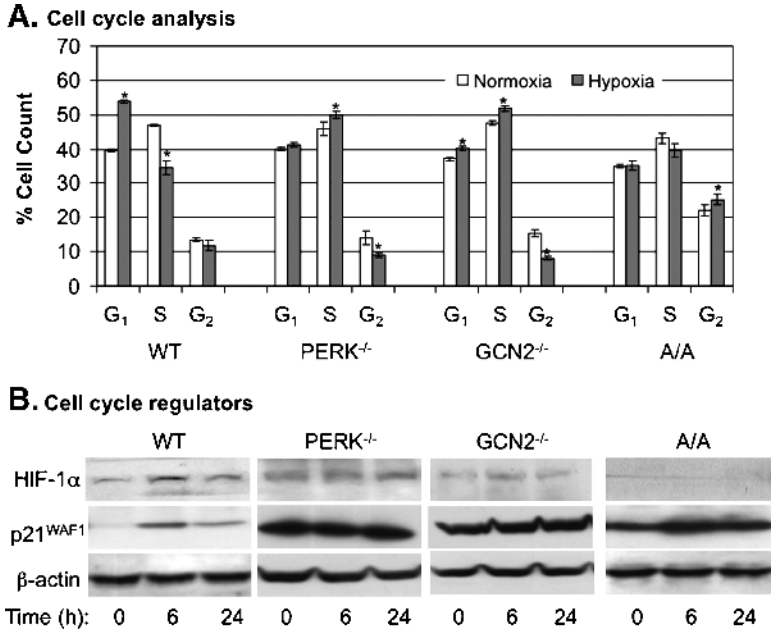

Figure 2. Hypoxia induces G_1 arrest in MEF^{WT} but not in $MEF^{PERK-/-}$, $MEF^{GCN2-/-}$, and $MEF^{A/A}$ cells. (A) The cells were treated with hypoxia for 12 hours before the cell cycle analysis using flow cytometry. The data were analyzed by ModFit software. The bars represent the means of three independent experiments. *$P < .05$ hypoxia *versus* normoxia. (B) The cells were treated with hypoxia for the time points as indicated, and the protein levels of HIF-1α and $p21^{WAF1}$ were analyzed by Western blot.

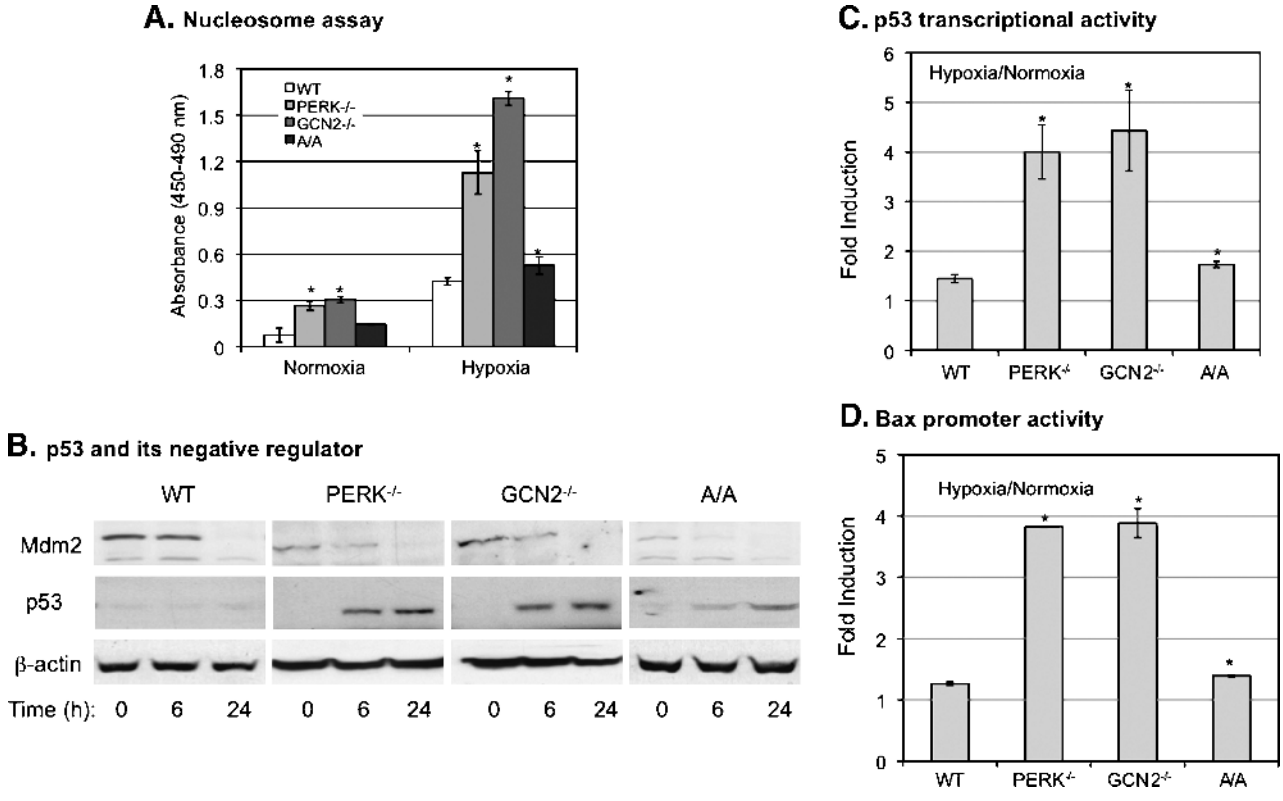

Figure 3. Both PERK and GCN2 regulate p53 signaling cascade and apoptosis in hypoxia. MEF^{WT}, $MEF^{PERK-/-}$, $MEF^{GCN2-/-}$, and $MEF^{A/A}$ cells were used in the experiments. (A) The cells were exposed to normoxia or hypoxia for 36 hours before apoptotic assays by detecting cleaved histone/DNA complex. The bars represent the means of three independent experiments. *$P < .05$ mutant *versus* wild type under normoxia or hypoxia. (B) The cells were treated with hypoxia for the time points as indicated and the protein level of Mdm2 was analyzed by Western blot. (C and D) The cells were cotransfected with p53 luciferase reporter plasmid or Bax luciferase reporter plasmid. Renilla luciferase reporter plasmid was used to normalize the transfection efficiency. Twenty-four hours after transfection, the cells were exposed to normoxia or hypoxia for 24 hours, and the activities of p53 and Bax were analyzed by luciferase assay. The bars represent the means of three independent experiments. *$P < .05$ mutant *versus* wild type.

to hypoxic stress, and then apoptotic cell death was analyzed by examining the cleavage of histone-DNA complex. Our data showed that higher levels of nucleosome contents were displayed in $MEF^{PERK-/-}$ and $MEF^{GCN2-/-}$ cells than the levels in MEF^{WT} and $MEF^{A/A}$ cells (Figure 3*A*). The result indicates that both PERK and GCN2 are involved in the regulation of apoptotic cell death in hypoxia.

Because p53 plays a central role in regulation of apoptosis during hypoxia [31–34], we determined whether PERK and GCN2 would protect cells from hypoxia-induced apoptosis via regulating p53 signaling pathway. Our data demonstrated that p53 was increased in the four cell lines on hypoxic stress (Figure 3*B*). However, the maximal inducibility of p53 was achieved in $MEF^{PERK-/-}$ and $MEF^{GCN2-/-}$ cells (Figure 3*B*). At the same time, the expression of a p53 negative regulator, Mdm2, was decreased in all cell lines independent of eIF2α phosphorylation levels (Figure 3*B*). These results suggest that activation of PERK and GCN2 inhibits hypoxia-induced activation of p53 signaling pathway independent of Mdm2. To confirm the results that the maximal inducibility of p53 was achieved in $MEF^{PERK-/-}$ and $MEF^{GCN2-/-}$ cells, we used p53 transcriptional activity–regulated luciferase expression system to determine p53 DNA binding ability. Our data showed that p53 transcriptional activity was up to 4.0 ± 0.5- and 4.4 ± 0.8-folds in $MEF^{PERK-/-}$ and $MEF^{GCN2-/-}$ cells, whereas the activity was only up to 1.4 ± 0.1- and 1.7 ± 0.1-folds in MEF^{WT} and $MEF^{A/A}$ cells, respectively, in hypoxia (Figure 3*C*). The promoter activity of a p53 downstream gene, *Bax*, was also upregulated more in $MEF^{GCN2-/-}$ and $MEF^{PERK-/-}$ cells than in MEF^{WT} and $MEF^{A/A}$ cells (Figure 3*D*). These results suggest that the lowered viability in $MEF^{PERK-/-}$ and $MEF^{GCN2-/-}$ cells was due to the upregulated apoptosis, which was mediated by p53 signaling pathways.

PERK, GCN2, and Phosphorylated eIF2α Promote Recovery of Cells from Hypoxia

The ability to recover from stress is also an important property to measure the regulation of survival and growth of cells after exposure to a stress. We determined whether PERK/GCN2–mediated eIF2α phosphorylation affects the ability of cells to recover from hypoxia. Clonogenic assay was used to measure survival and recovery of cells from hypoxic stress. The MEF^{WT}, $MEF^{PERK-/-}$, $MEF^{GCN2-/-}$, and $MEF^{A/A}$ cells were exposed to hypoxia for 24 hours and then cultured under normal conditions for 6 days. Our data showed that, compared with MEF^{WT} cells, the survival rates after hypoxia were reduced 50.5 ± 8.4% and 50.0 ± 2.9% for $MEF^{PERK-/-}$ and $MEF^{GCN2-/-}$ cells, respectively (Figure 4*A*). The survival rate of $MEF^{A/A}$ cells was reduced only by 5.9 ± 5.9% (Figure 4*A*). Noticeably, the sizes of $MEF^{A/A}$ colonies were much smaller than the other three cell lines (Figure 4*B*), indicating that eIF2α phosphorylation was required for cell growth during recovery but not required for cell survival on hypoxia. These data demonstrate that partial reduction of eIF2α phosphorylation (PERK or GCN2 knockout) decreases cell recovery by lowering survival rate, whereas total elimination of eIF2α phosphorylation (eIF2α S51A mutation) decreases cell recovery by lowering cell growth rate

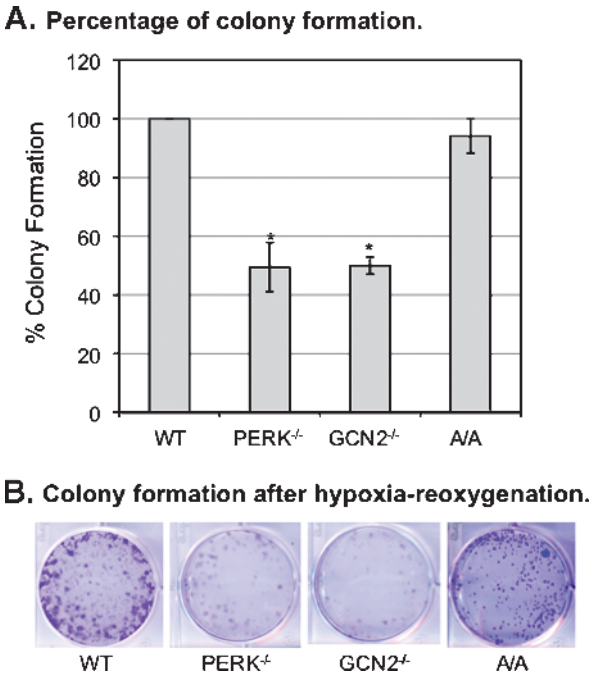

Figure 4. Both PERK and GCN2 mediate recovery of cells from hypoxic stress. MEF^{WT}, $MEF^{PERK-/-}$, $MEF^{GCN2-/-}$, and $MEF^{A/A}$ cells were exposed to hypoxia for 24 hours. Cells were collected and stained by Trypan blue. Nonapoptotic cells were counted. Five thousand cells were replated and cultured under normoxia for 6 days. Cells were then fixed by methanol for 10 minutes at −20°C and stained by 1% crystal violet (25% methanol) for 10 minutes, washed by distilled water, and dried. (A) The colonies with a size greater than 0.5 mm were counted. The degree of colony formation was expressed as percentage of MEF^{WT} cells. The bars represent the means of three independent experiments. $^*P < .05$ mutant *versus* wild type. (B) The plates were photographed using microscopy equipped with a Nikon digital camera.

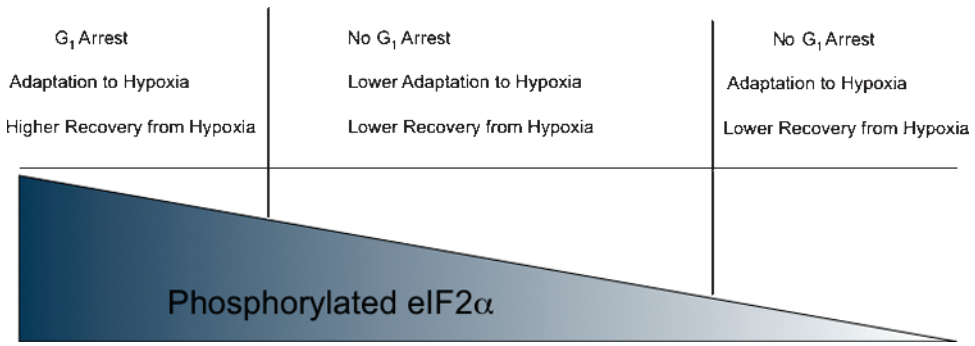

Figure 5. The model for the roles of eIF2α phosphorylation in cell fate determination.

during reoxygenation after hypoxic stress. These results suggest that the hypoxia-induced activation of PERK/GCN2 and eIF2α phosphorylation coordinatively regulate cell growth and apoptosis after hypoxic stress.

Discussion

A hypoxic microenvironment in solid tumors often correlates with tumor progression and therapy resistance [35]. HIF-1α is a key regulator in response to hypoxia and has been shown to regulate angiogenesis, metabolic adaptation, proliferation, apoptosis, and invasion [9–12,36–39]. Hypoxia increases cancer cell–induced lymphatic endothelial cell invasion and migration [40], whereas it decreases macromolecular synthesis and slows down cell proliferation [2,41]. Prolonged hypoxia also induces an energy-depleting response that activates mammalian target of rapamycin–signaling network [42]. Recent studies show that hypoxia activates PERK, which phosphorylates eIF2α, reduces protein synthesis, and contributes to hypoxic adaptation [3–6,26,28]. However, in hypoxic cells lacking PERK, the phosphorylation of eIF2α on Ser 51 is still upregulated [26], which indicates that there could be other EIF2AKs activated in hypoxia. In this study, we demonstrate that similar to $MEF^{PERK-/-}$ cells, the phosphorylation of eIF2α in $MEF^{GCN2-/-}$ cells was also delayed in hypoxia (Figure 1*A*), suggesting that GCN2 is also involved in the phosphorylation of eIF2α in response to hypoxia. Besides PERK and GCN2, HRI and PKR can also phosphorylate eIF2α. Although PKR has been shown not to induce eIF2α phosphorylation under hypoxia [26], we cannot rule out that HRI is also involved in hypoxia-induced eIF2α phosphorylation.

Our results suggest that reduction of eIF2α phosphorylation by PERK or GCN2 knockout sensitized the cells to hypoxia-induced cell death (Figures 1 and 4), but elimination of eIF2α phosphorylation by eIF2α S51A knock-in had no effects on the cell survival under the same conditions (Figures 1 and 4). These results disagree with the previous *in vitro* study indicating that abolishing eIF2α phosphorylation reduces cell survival in hypoxia [3]. However, our results agree with the *in vivo* data in the same report, which shows that the tumor volume from $MEF^{A/A}$ cells is much larger than the tumors from $MEF^{PERK-/-}$ cells but is smaller than the tumors from MEF^{WT} cells. Because tumor hypoxia is transient *in vivo* and reoxygenation also affects tumor survival and growth, we further confirm our *in vitro* data by investigating the survival and recovery on hypoxia-reoxygenation in the four cell lines. Our data from the viability and colonogenic assays indicate that partial reduction of eIF2α phosphorylation by knocking out either PERK- or GCN2-promoted cell death but did not affect cell growth on hypoxia-reoxygenation (Figure 4). In contrast, totally abolished eIF2α phosphorylation did not influence cell survival but promoted cell growth on hypoxia-reoxygenation (Figure 4). These results suggest that PERK/GCN2 knockout affects cell growth and death on hypoxia-reoxygenation through differential mechanism as eIF2α S51A mutation does. The controversial observation with the previous *in vitro* data [3] may be due to the experimental conditions. We used moderate hypoxia (~1% oxygen), which is close to the physiological condition in solid tumor [43,44]. However, the previous study was performed under extreme hypoxia (<0.02% oxygen) [3].

To further analyze the mechanism for EIF2AKs and eIF2α phosphorylation–regulated tumor progression in hypoxia, we analyzed their roles in regulating the expressions of several key hypoxia-induced factors, such as HIF-1α and p53 [25,31–34,39,45]. The correlations among the gene expression patterns, cell cycle arrest, and apoptosis were also studied. Our data showed that the hypoxia-induced eIF2α phosphorylation is critical in the regulation of the inducibility and expression of HIF-1α and $p21^{WAF1}$ (Figure 2). However, only the inducibilities, but not the levels, of HIF-1α and $p21^{WAF1}$ expression were correlated to hypoxia-induced cell cycle arrest (Figure 2). Whereas PERK and GCN2 are required for hypoxia-induced HIF-1α/$p21^{WAF1}$ expression, they suppress hypoxia-induced activation of p53 (Figure 3). Elimination of PERK or GCN2 activity also sensitizes the cells to hypoxia-induced apoptosis (Figure 3). Interestingly, totally abolishing eIF2α phosphorylation has no effects on p53 activation and apoptosis (Figure 3), but affects HIF-1α/$p21^{WAF1}$ inducibility and G_1 arrest after hypoxic stress (Figure 2). The mechanism could be due to the extremely low expression of HIF-1α in both normoxia and hypoxia (Figure 2*B*), which plays a critical role in regulation of hypoxia-induced cell cycle arrest [46]. On the basis of these results, we propose that PERK/GCN2–mediated eIF2α phosphorylation serves as a "switch" that determines the fate of cells after hypoxic stress (Figure 5). A lower level of eIF2α phosphorylation "turns on" a death signal by inhibiting G_1 arrest and promoting apoptosis through activating p53 signal cascade. In contrast, higher levels of eIF2α phosphorylation "switch" the death signal to an adaptive signal by promoting G_1 arrest and reducing apoptosis through activating the HIF-1α/$p21^{WAF1}$ signaling pathway. Our proposed model has the potential to be used to develop new therapeutics for cancer treatment by targeting PERK/GCN2–meidated eIF2α phosphorylation.

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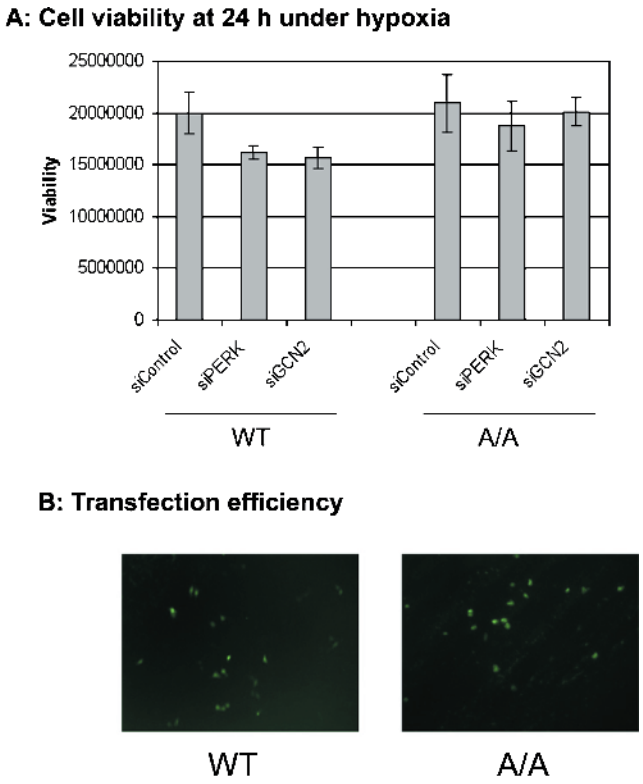

Figure W1. (A) siRNAs were transfected into MEF^{WT} and MEF$^{A/A}$ cells to knock down PERK or GCN2. The transfected cells were exposed to hypoxia for 24 hours and viability was measured. (B) GFP vector was transfected into MEF^{WT} and MEF$^{A/A}$ cells to show the transfection efficiency.