

GSH depletion and consequent AKT inhibition contribute to the Nrf2 knockdown-induced decrease in proliferation in glioblastoma U251 cells

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Abstract. Nuclear factor erythroid 2-related factor 2 (Nrf2), a pivotal transcription regulator that controls the expression of numerous antioxidant and cytoprotective genes, was recently defined as a proto-oncogene. However, the role and mechanism of Nrf2 in glioma pathoetiology remain unclear. In the present study, we first evaluated the expression patterns of Nrf2 in normal human astrocytes and 3 glioblastoma (GBM) cell lines (U251, U87 and A172) and found that all 3 GBM cell lines overexpressed Nrf2, with the highest level observed in the U251 cells. We further assessed the biological effects of Nrf2 in U251 cells by specific knockdown of Nrf2 using lentivirus-mediated RNA interference. We discovered that Nrf2 deficiency led to a decrease in U251 cell proliferation and caused intracellular redox imbalance [diminished glutathione (GSH) levels and increased reactive oxygen species (ROS) levels]. Both *N*-acetylcysteine and glutathione monoethyl ester (GMEE) supplementation completely eliminated the increased levels of ROS that were present in the Nrf2-deficient U251 cells. However, only GMEE supplementation both reversed Nrf2 deficiency-induced cell growth arrest and restored intracellular GSH levels. Moreover, AKT and ERK1/2

signaling were both impaired in the Nrf2-knockdown U251 cells, but GMEE supplementation restored AKT signaling but not ERK1/2 signaling, and blocking AKT signaling with an AKT-specific inhibitor greatly diminished the GMEE-induced Nrf2-deficient cell proliferation. In conclusion, our findings revealed novel functions for Nrf2 in the regulation of redox status and cell proliferation, and that intracellular GSH levels and AKT signaling are required for this process, a new viewpoint by which to comprehend the role and underlying mechanism of Nrf2 in tumorigenesis.

Introduction

Oxidative stress in cancer is one of the most important metabolic stresses caused by both extrinsic (reperfusion of a hypoxic microenvironment) and intrinsic (uncoupled mitochondrial activity) factors (1,2). From the viewpoint of carcinogenesis, oxidative stress is a double-edged sword, since it promotes genetic alterations through DNA damage to drive malignant progression while also causing cell damage and inducing apoptosis (3,4). Under physiological conditions, cells possess molecular mechanisms that enable them to adapt to these stresses. In multicellular organisms, adaptive responses to oxidative stresses are regulated by nuclear factor erythroid 2-related factor 2 (Nrf2), a master transcription factor of many antioxidant genes and phase II detoxifying enzymes (5). Moreover, recent studies have demonstrated that cancer cells can also use Nrf2 and its target genes to adapt to multiple stresses, which is beneficial for cancer cell survival, leading to malignant progression and lethal characteristics such as invasion, metastasis and chemotherapy resistance (6). Therefore, some researchers have pointed out that under these conditions, Nrf2 can be defined as a proto-oncogene.

Transcription factor Nrf2, a critical element in the survival of healthy cells in response to oxidative stress, belongs to the Keap1-Nrf2-antioxidant response element (ARE) signaling pathway and upregulates many ARE-containing genes such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), aldo-keto reductase 1C1 (AKR1C1) and thioredoxin (Trx) (7). In addition to these enzymes, these proteins also include, for example, those involved in the

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Abbreviations: AKT, protein kinase B; DCF, dichlorofluorescein; ERK1/2, extracellular signal-regulated kinase 1/2; GBM, glioblastoma; GCLC, catalytic subunit of γ -glutamylcysteinyl ligase; GMEE, glutathione monoethyl ester; GR, glutathione reductase; GSH, glutathione; HO-1, heme oxygenase-1; NAC, *N*-acetylcysteine; NHA, normal human astrocytes; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; shRNA, short hairpin RNA; STAT3, signal transducers and activation of transcription 3

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biosynthesis and regeneration of glutathione (GSH), a very effective scavenger of reactive oxygen species (ROS) and electrophiles, such as heavy and light chains of γ -glutamyl cysteinyl ligase (8), the x-CT (subunit of the cystine/glutamate transporter) component of the cystine/glutamate exchange transport system (9), the glutathione reductase (GR) and the GSH synthetase (10,11).

In the present study, we first assessed the expression patterns of Nrf2 in normal human astrocytes and 3 GBM cell lines, and subsequently silenced Nrf2 expression in the GBM cell line U251 using RNA interference technology. Functional analysis demonstrated that Nrf2 knockdown led to a decrease in U251 cell proliferation, as well as resulted in intracellular redox imbalance (decreased levels of GSH and increased levels of ROS). In addition, suppression of Nrf2 also resulted in the impairment of the AKT and ERK1/2 signaling pathways. Finally, we ascertained that exogenous supplementation of Nrf2-knockdown cells with glutathione monoethyl ester (GMEE; a membrane-permeable derivative of GSH that can supplement cellular GSH levels) restored their intracellular GSH levels, cell proliferation defects and AKT inhibition, confirming that GSH depletion and AKT pathway inhibition are critical for GBM cell proliferation. Our findings may shed light on the role and underlying mechanism of Nrf2 in the regulation of glioma proliferation via GSH depletion and consequent AKT inhibition, a new viewpoint by which to comprehend the role and mechanism of Nrf2 in glioma pathoetiology.

Materials and methods

Reagents and antibodies. *N*-acetylcysteine (NAC) was obtained from Beyotime (Shanghai, China). GMEE (sc-203974), AKT inhibitor IV (sc-203809) and AG490 (sc-202046) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). U0126 (cat. #9903) was purchased from Cell Signaling Technology (CST; Danvers, MA, USA). Antibodies against phospho-AKT (Ser473), AKT, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-STAT3 (Tyr705), STAT3, histone H3 and β -actin were purchased from CST. Antibodies against GR, GCLC and Nrf2 were obtained from Santa Cruz Biotechnology.

Cell culture. Human GBM cell lines A172, U87 and U251 were obtained from the Cell Bank of Shanghai, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37°C in 5% CO₂/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco, Los Angeles, CA, USA).

Normal human astrocytes (NHAs) were obtained from ScienCell (San Diego, CA, USA) (cat. 1800). NHAs were cultured in astrocyte medium (cat. 1801; ScienCell) and cultured following the manufacturer's instructions.

Lentivirus-mediated RNA interference. For lentivirus-mediated silencing of Nrf2, the lentiviral particles and Polybrene were purchased from GenePharma (Shanghai, China). The short hairpin RNA (shRNA) sequence that effectively targets

human Nrf2 was: 5'-GCAGTTCAATGAAGCTCAACT-3'. A non-silencing shRNA, with the scrambled sequence 5'-TTCTC CGAACGTGTCACGT-3', was used as the negative control. The lentiviral particles containing Nrf2-specific shRNA or scrambled shRNA were named Nrf2i or Sc, respectively. Cell transfection was performed as per the manufacturer's instructions. Briefly, the cells were plated in a 6-well plate to reach 30-50% confluence on the day of transfection. After the addition of Polybrene at 4.5 μ g/ml, the lentiviral particles were used to transfect the cells. Transfection was continued for 24 h followed by a 24-h recovery period in complete medium. For the selection of cells with stable transfection, cells were grown under selective pressure by puromycin (2.0 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA) for up to 2 weeks.

Quantitative real-time PCR. Total RNA was extracted using a modified TRIzol one-step extraction method (Tiangen, Beijing, China), and then the quality of total RNA was assessed by spectrophotometer analysis (OD_{260/280}:1.8-2.2). An amount 400 ng RNA of total RNA was reverse-transcribed using First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). The cDNA obtained was amplified immediately using the following primers: for Nrf2, 5'-TCAGCGACGGA AAGAGTATGA-3' and 5'-CCACTGGTTTCTGACTGGA TGT-3'; for NQO1, 5'-ATGGTTCGGCAGAAGAGC-3' and 5'-GGAAATGATGGGATTGAAGT-3'; for HO-1, 5'-TCTCC GATGGGTCTTACACTC-3' and 5'-GGCATAAAGCCCT ACAGCAACT-3'; for GCLC, 5'-AGACATTGATTGTCTG CTG-3' and 5'-TGGTCAGACTCATTAGCA-3'; for GR, 5'-TTTGTCTCGGTCTTTCGGGG-3' and 5'-CCAGGATCT ATGGCACCGTC-3'; and for GAPDH, 5'-GAAATCCCATC ACCATCTTC-3' and 5'-GGACTCCACGACGTACTCA-3'. The amplification and data acquisition were carried out on a Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) using FastStart Universal SYBR-Green Master (Roche, Mannheim, Germany). The conditions consisted of pre-denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The PCR amplification efficiency of each gene was established by means of calibration curves. All samples were analyzed in triplicate. The results were analyzed using the 2^{- $\Delta\Delta$ C_q} method. The data are expressed as the relative mRNA levels and were normalized to GAPDH, a commonly used control.

Western blot analysis. To prepare the total proteins, cells were lysed in cold RIPA lysis buffer containing a 0.5% phosphatase inhibitor and 1% phenylmethylsulphonyl fluoride (both from Beyotime, Jiangsu, China). To prepare the nuclear and cytoplasmic proteins, cells were lysed using a nuclear and cytoplasmic protein extraction kit (Beyotime) as per the manufacturer's instructions. Protein content was quantified with the Bradford reagent (Coomassie Plus Protein Assay Reagent; cat. #23238) (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by 8-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk in TBS-1% Tween-20 (TBST) for 2 h, the membranes were immunoblotted with primary antibodies: 1:500 anti-Nrf2 (cat. #sc-13032; 100 kDa), 1:500 anti-GCLC

(cat. #sc-22755; 73 kDa), 1:500 anti-GR (cat. #sc-32886; 50-65 kDa) (all from Santa Cruz Biotechnology) 1:1,000 anti-phospho-AKT (cat. #4060; 60 kDa), 1:1,000 anti-AKT (cat. #4691; 60 kDa), 1:1,000 anti-phospho-ERK1/2 (cat. #4370; 42.44 kDa), 1:1,000 anti-ERK1/2 (cat. #4695; 42.44 kDa), 1:1,000 anti-phospho-STAT3 (cat. #9145; 79.86 kDa), 1:1,000 anti-STA3 (cat. #12640; 79.86 kDa), 1:2,000 anti- β -actin (cat. #4970; 45 kDa) and 1:1,000 anti-histone H3 (cat. #4499; 17 kDa) (all from CST). After removal of the primary antibodies, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature and visualized using the enhanced chemiluminescent (ECL) detection reagent from Pierce. Immunoreactive protein bands were detected with Tanon 5200 chemiluminescence imaging system (Shanghai, China).

Doubling time. Aliquots of 5×10^4 cells were seeded in 35-mm plastic dishes in culture medium. The number of cells was counted in triplicate at 24-h intervals for 4 days using a particle distribution counter CDA-500 (Sysmex, Kobe, Japan). The doubling time of the cell population was estimated from the exponential growth phase.

Cell proliferation analysis. Cell proliferation was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) as per the manufacturer's protocol. One day prior to treatment, 1,000 cells/well were plated in a 96-well tissue culture plate in a total volume of 200 μ l/well and were cultured for 1-4 days, respectively. Subsequently, 10 μ l of CCK-8 solution was added and the cells were further incubated for 1 h at 37°C in 5% CO₂/95% air. Then, the absorbance was measured at 450 nm using an ELISA microplate reader (Bio-Rad, Hercules, CA, USA). At least 6 technical replicates were used for each condition.

Colony formation analysis. Colony formation analysis was carried out 9 days after lentivirus transfection. U251 cells were plated into 6-well plates (300 cells/well) and cultured for 9 days. The medium was updated every 3 days. After 9 days of culture, the cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min, and were stained with freshly prepared crystal violet staining solution for 20 min. Colony formation was observed through a light microscope and a colony count was performed.

Assessment of total GSH content. Cells were collected and lysed with protein detergent S solvent. The total GSH was determined by commercially available Total Glutathione Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). All procedures completely complied to the manufacturer's instructions. The protein concentration was estimated by Coomassie Plus Protein Assay Reagent (Pierce).

Assessment of intracellular ROS. The level of intracellular ROS was quantified using the Reactive Oxygen Species Assay kit (Beyotime Institute of Biotechnology). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is oxidized by ROS in viable cells to dichlorofluorescein (DCF) which is highly fluorescent at 530 nm. The cells were washed 3 times with PBS. DCFH-DA, diluted to a final concentration

of 10 mM, was added and incubated for 30 min at 37°C in the dark. After being washed 3 times with PBS, the relative levels of fluorescence were quantified by a multidetection microplate reader (485 nm excitation and 535 nm emission).

Statistical analysis. Data bars correspond to the mean values \pm standard deviations (SD) of minimum triplicate experiments. The statistical significance of the differences was assessed using one-way analysis of variance (ANOVA) or unpaired two-tailed t-tests; the probability values of <0.05 and <0.01 were deemed statistically significant and very significant, respectively, for all analyses.

Results

Nrf2 is overexpressed in GBM cell lines. In a previous study, we found that the expression level of Nrf2 was positively correlated with the tumor grade in glioma tissues (23). In the present study, we used western blot analyses to examine Nrf2 levels in various GBM cell lines and normal human astrocyte (NHA) cell line. As shown in Fig. 1A, western blot analysis revealed that all 3 cell lines, U251, U87 and A172, had a higher Nrf2 level than that of the NHA cell line both in the nuclear fraction and in the cytosol fraction. To be noted, there was no detectable band in the nuclear fraction of the NHA cell line, while Nrf2 was accumulated most abundantly in the nuclear fraction of the U251 cells. Consistent with the aforementioned results, similar patterns were observed in the mRNA levels of Nrf2. All three human GBM cell lines (U251, U87 and A172) had increased relative mRNA levels of Nrf2 compared with the NHA cell line (Fig. 1B).

As a transcription factor, overexpression of Nrf2 may cause expression of downstream genes; thus, we tested the mRNA levels of NQO1 and HO-1 using real-time PCR. As shown in Fig. 1B, the highest Nrf2 level was observed in the U251 cells, as well as the mRNA levels of HO-1 and NQO1. Therefore, Nrf2 is overexpressed and transcriptionally active, which may cause overexpression of downstream genes in multiple GBM cell lines.

We also tested the doubling times for U251, U87 and A172 cells (~8, 24 and 48 h, respectively). It was determined that U251, with the highest Nrf2 expression level, also had the fastest proliferation speed of all 3 cell lines (data not shown). In accordance with this, we chose the U251 cell line for subsequent genetic intervention.

Nrf2 deficiency leads to oxidative stress and redox imbalance in GBM cells. To generate the stable Nrf2-knockdown model, the U251 cells were transfected with lentivirus-mediated shRNA encoding Nrf2-targeting shRNA (Nrf2i) or scrambled control (Sc) shRNA. Clearly, transfection with Sc shRNA did not affect the expression of Nrf2 as the Nrf2 mRNA transcripts and protein expression in the Sc group were similar to that in the control group (Fig. 2A and B). In contrast, the levels of Nrf2 mRNA transcripts in the U251-Nrf2i cells were significantly decreased by ~80% ($P < 0.01$) (as shown in Fig. 2A), compared with the non-treated or Sc shRNA-treated U251 cells. Additionally, western blot analysis revealed that the Nrf2 expression was also significantly decreased in the Nrf2i group (Fig. 2B). These results indicated

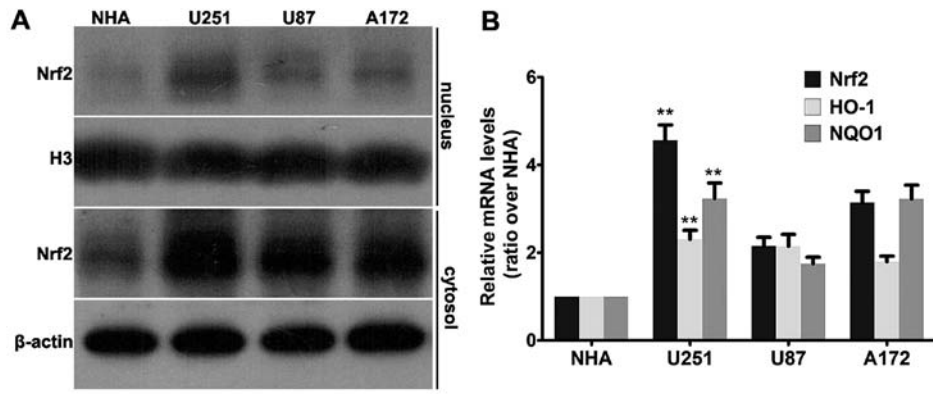


Figure 1. Human GBM cell lines accumulate Nrf2. (A) Western blot analysis of nuclear and cytosolic fractions for Nrf2 protein levels revealed that 3 GBM cell lines (U251, U87 and A172) accumulated activated Nrf2 either in the cytosol or in the nuclei compared with a normal human astrocyte (NHA) cell line. β -actin and H3 were used as loading controls. (B) Real-time quantitative PCR analysis revealed that human GBM cell lines (U251, U87 and A172) exhibited increased relative mRNA levels of Nrf2 compared with the NHA group. The relative mRNA levels of Nrf2 target genes HO-1 and NQO1 were also increased in comparison with the mRNA levels of Nrf2. Bar graphs were obtained from real-time quantitative PCR analysis and are expressed as ratios over the NHA group, respectively; ** $P < 0.01$ vs. the NHA group.

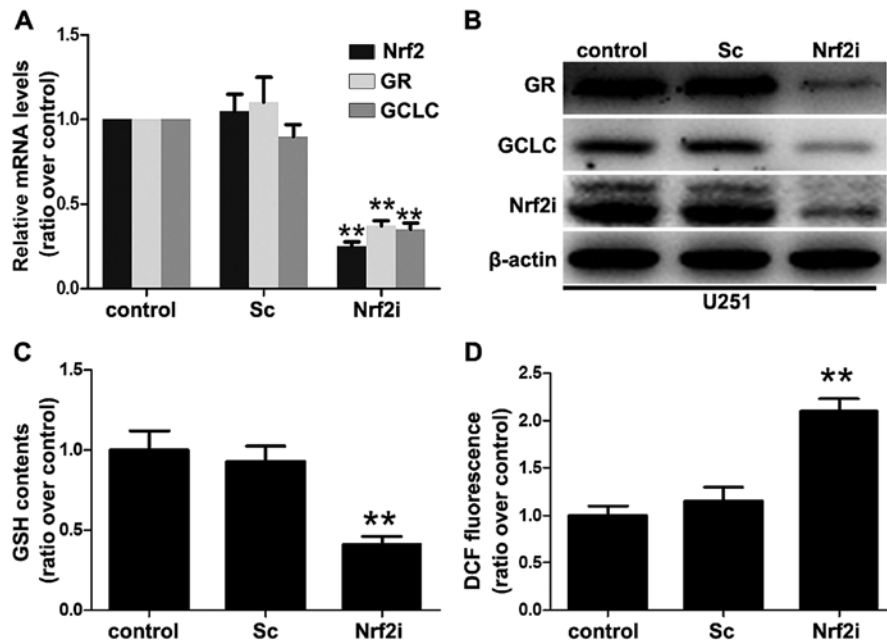


Figure 2. U251 cells deficient in Nrf2 undergo oxidative stress as a result of decreased GSH levels. (A) Relative mRNA levels of Nrf2, GCLC and GR ascertained the inhibition of Nrf2 in U251 cells. Bar graphs were obtained from real-time quantitative PCR analysis and are expressed as the ratios over the control group, respectively; ** $P < 0.01$ vs. the control group. (B) The protein expression of Nrf2, GCLC and GR was determined by western blot analysis using β -actin as the loading control. (C) GSH levels in non-treated (control), scrambled control (Sc) shRNA and Nrf2 shRNA (Nrf2i)-treated U251 cells were determined. The control value was 20.83 ± 0.93 nmol/mg; ** $P < 0.01$ vs. the control group. (D) The relative levels of reactive oxygen species (ROS) in non-treated (control), Sc shRNA and Nrf2 shRNA (Nrf2i)-treated U251 cells. ROS generation was determined using the fluorescence probes DCFH-DA. Values are expressed as the mean \pm SD from 3 experiments; ** $P < 0.01$ vs. the control group.

that lentivirus-mediated shRNA effectively and specifically suppressed Nrf2 expression in the U251 cells.

As a transcription factor, knockdown of Nrf2 may suppress the expression of its regulated genes, for example GCLC and GR, which are also known as GSH generating enzymes [catalytic subunit of GCL (GCLC) for *de novo* GSH biosynthesis and GR for GSH regeneration]. Thus, we determined the GCLC and GR mRNA levels using real-time PCR to evaluate the function of Nrf2. As shown in Fig. 2A, the Nrf2 mRNA level was suppressed in the U251 cells accompanied by the decreased mRNA levels of GCLC and GR. Similar patterns

were observed in the immunoblot analysis of the proteins GCLC and GR (Fig. 2B).

Since Nrf2 is the GSH-generation key regulator (as indicated above), several *in vivo* studies have shown that an Nrf2 deficiency results in oxidative stress and redox imbalance (decreased levels of GSH and increased levels of ROS) (2,3). To characterize this effect, we determined the GSH levels in the non-treated, Sc shRNA-treated and Nrf2 shRNA (Nrf2i)-treated cells (Fig. 2C). Consistent with the results obtained with decreased GCLC and GR expression, we found that the GSH levels in the Nrf2-knockdown U251 cells were

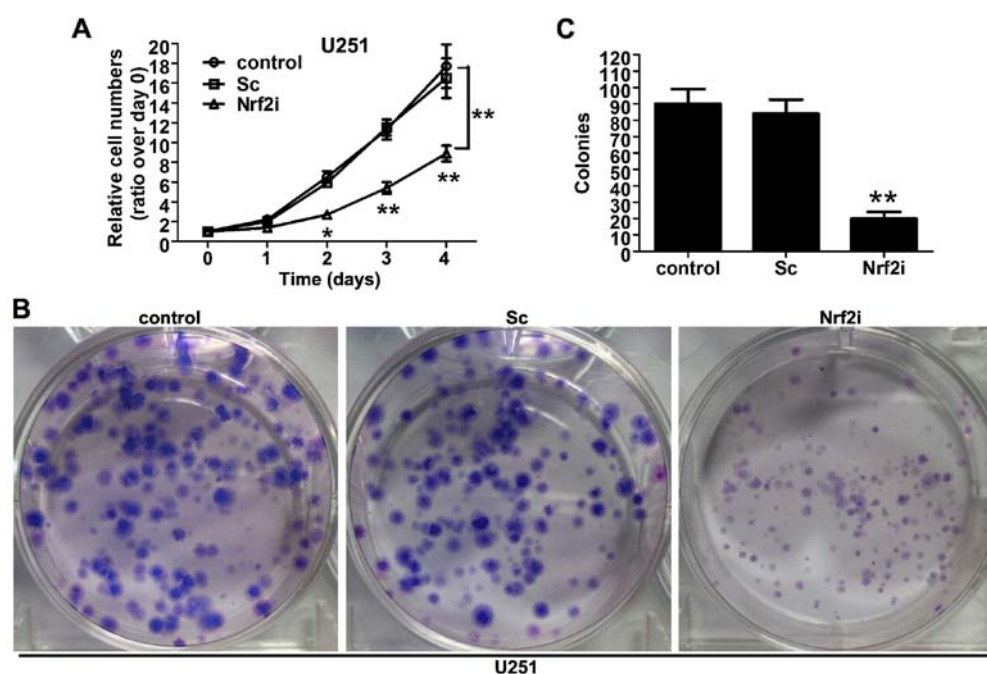


Figure 3. U251 cell proliferation is decreased following the disruption of Nrf2. (A) Cells were cultured for 4 days and cell proliferation was assessed every 24 h by CCK-8 assay; * $P < 0.05$, ** $P < 0.01$ vs. the control group. (B) The size and number of colonies in the non-treated (control), scrambled control (Sc) shRNA and Nrf2-shRNA (Nrf2i)-treated U251 cells were assessed and recorded under a light microscope. The representative images are from one of 3 independent experiments performed. (C) Statistical analysis of the number of colonies in the control, Sc and Nrf2i groups. Data are presented as the mean \pm SD of 3 independent experiments; ** $P < 0.01$ as compared with the control group.

significantly decreased (decreased by 70% compared with the Sc and control groups). To further correlate these results with those related to cellular redox status, we determined the intracellular levels of ROS using DCF-DA dye, which emits green fluorescence in the presence of ROS. As shown in Fig. 2D, U251-Nrf2i cells exhibited a 117.4% increase in ROS production in comparison to the control group (Fig. 2D). Collectively, these data indicated that lentivirus-mediated shRNA silencing led to oxidative stress as a result of decreased GSH levels and redox imbalance in the GBM cells.

U251 cell proliferation is impaired with the disruption of Nrf2. To investigate the effect of Nrf2 silencing on cell proliferation, the cell viability was observed using CCK-8 assay. As shown in Fig. 3A, the growth curve of the Nrf2i group (Nrf2 shRNA-treated U251 cells) began to decline from the second day, compared with the control group (non-treated) and Sc group (Sc-treated U251 cells). The decrease reached 37.1% ($P < 0.01$) and 45.2% ($P < 0.01$) on the third and fourth day, respectively, compared with the Sc and control groups, whereas no significant difference relating to cell viability was observed between the Sc and control groups. To further ascertain the results of the CCK-8 assay, we conducted the colony formation assay to evaluate the long-term effect of Nrf2 knockdown on U251 cell proliferation. As shown in Fig. 3B, the size of the independent colonies was much smaller in the Nrf2i group than that in the control group and Sc group. Furthermore, the number of colonies that formed in the Nrf2i group was significantly decreased ($P < 0.01$), compared with the control and Sc groups (Fig. 3C). The aforementioned data indicate that Nrf2 knockdown evidently impaired the proliferation of U251 cells.

Exogenous supplementation with GMEE, but not NAC, rescues the phenotypic defects associated with Nrf2 deficiency. To further confirm the mechanism of Nrf2 knockdown-induced inhibition of GBM cell proliferation, we wondered whether exogenous supplementation of the Nrf2-knockdown cells with NAC or GMEE (a membrane-permeable derivative of GSH that can supplement cellular GSH levels) may restore their intracellular redox status and cell proliferation. When Nrf2-knockdown cell cultures were supplemented on day 1 to day 4 with NAC, GMEE or vehicle (PBS) at various concentrations (see Fig. 4A and B), we found that supplementation with GMEE, but not NAC, restored cell proliferation in the Nrf2-knockdown cells for the most part, although the treated cells did not reach the same levels in proliferation as was observed in the control and Sc groups (Fig. 4B). GMEE supplementation also restored the cellular GSH levels that were decreased in the Nrf2-deficient U251 cells (Fig. 4C). Notably, we found that supplementation of Nrf2-deficient cells with NAC, a precursor of GSH, did not improve cell proliferation (Fig. 4A), even though it completely eliminated the high levels of ROS that were otherwise present in the Nrf2-knockdown cells (Fig. 4D). These results collectively suggest that, in addition to squelching ROS, intracellular GSH levels are critical for the proliferation of Nrf2-knockdown cells.

AKT signaling is critical for GMEE-induced Nrf2-deficient U251 cell proliferation. To further delineate the mechanisms by which Nrf2 regulates cell proliferation, we examined the status of intracellular signal transduction pathways, such as AKT, ERK1/2 and STAT3, which are known to be important in cell proliferation. Since exogenous supplementation of Nrf2-deficient cells with GMEE did not increase cell

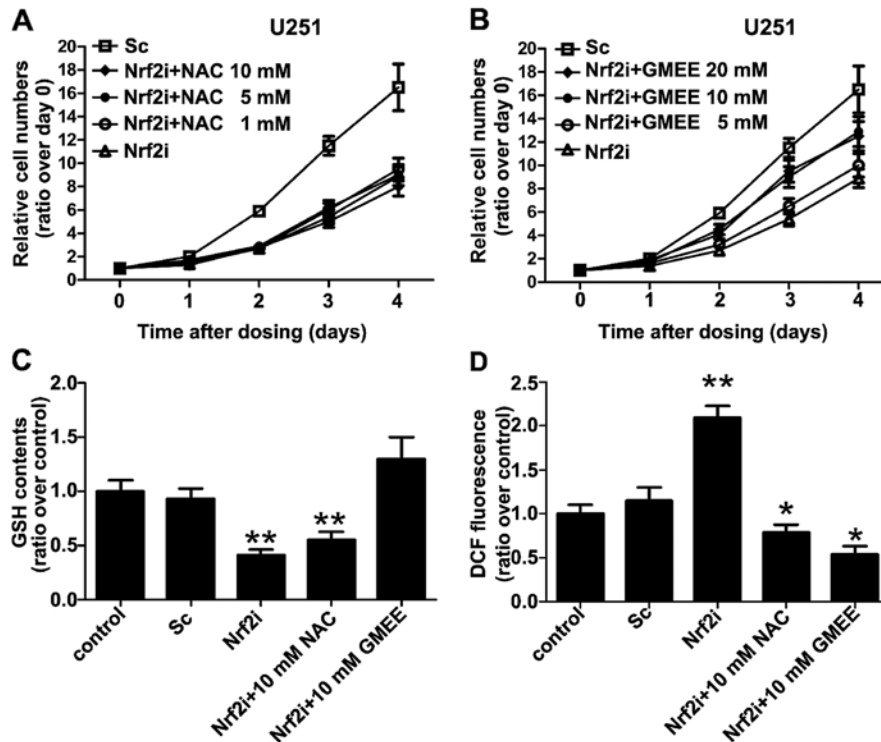


Figure 4. Exogenous supplementation with glutathione monoethyl ester (GME) restores Nrf2 deficiency-related phenotypic defects such as cell proliferation. (A and B) Supplementation with GME, but not NAC, mostly restored the arrest in cell proliferation noted in the Nrf2-knockdown cells, although the treated cells did not reach the same level as was observed in the Sc group. Cells were grown in the absence or presence of GME (5, 10 and 20 mM) and NAC (1, 5 and 10 mM) for 4 days and cell proliferation was assessed every 24 h by CCK-8 assay. (C) Supplementation of Nrf2-deficient U251 cells with GME, but not NAC, restored normal GSH levels that were decreased in the Nrf2-knockdown cells. Cells were grown in the absence or presence of 10 mM GME or 10 mM NAC. On day 4 the cells were washed with PBS, lysed and the GSH levels were determined; ** $P < 0.01$ as compared with PBS (vehicle)-treated control group. (D) Following supplementation of Nrf2-deficient U251 cells with GME or NAC, both were able to eliminate the increased levels of ROS that were present in the Nrf2-knockdown cells. Cells were grown in the absence or presence of 10 mM GME or 10 mM NAC. On day 4 the cells were washed with PBS, lysed and the relative levels of reactive oxygen species (ROS) generation were determined using the fluorescence probes DCFH-DA. Values are expressed as the mean \pm SD from 3 experiments; * $P < 0.05$, ** $P < 0.01$ as compared with the PBS (vehicle)-treated control group.

proliferation to the same extent as was observed in the counterpart (as shown in Fig. 4B), we reasoned that knockdown of Nrf2 in U251 cells may cause the dysregulation of a series of signal transduction pathways, but GME can only restore some, not all of them. In the present study, we analyzed the phosphorylation status and protein levels of AKT, ERK1/2 and STAT3 kinases in the Nrf2-deficient cells with or without GME supplementation by immunoblotting. As shown in Fig. 5A, Nrf2 depletion decreased the phosphorylation status as well as the protein levels of AKT. Nrf2 knockdown also significantly decreased the phosphorylation, but not the level of expression of ERK1/2 kinases (Fig. 5A), whereas the phosphorylation status and the protein levels of STAT3 exhibited no evident alteration in the Nrf2-deficient U251 cells. Moreover, the phosphorylation and expression levels of AKT kinase were restored when the Nrf2-deficient U251 cell cultures were supplemented with GME. However, the GME supplementation failed to restore the phosphorylation status of ERK1/2 kinases that were also dysregulated in the Nrf2-deficient cells. Collectively, Nrf2 deficiency caused the inhibition of the AKT and ERK1/2 pathways in U251 cell lines, but exogenous supplementation of GME only restored AKT but not ERK1/2 kinases.

To further verify the results of western blotting, we performed a set of experiments with the AKT-specific inhibitor, AKT inhibitor IV; the ERK inhibitor, U0126; and the STAT3

inhibitor, AG490. AKT inhibitor IV (5 μ M), U0126 (10 μ M) and AG490 (10 μ M) were used to interrupt the signaling transduction of AKT, ERK1/2 and STAT3 at concentrations that did not affect the cell viability when applied alone (data not shown). We found that inhibition of ERK1/2 and STAT3 had no significant effect on GME-induced Nrf2-deficient cell proliferation (Fig. 5C and D). In contrast, blocking AKT signaling with an AKT-specific inhibitor markedly blocked the Nrf2-deficient cell proliferation induced by GME (Fig. 5B). Collectively, these results strongly suggest that AKT signaling is important in GME-induced Nrf2-deficient cell proliferation.

Discussion

In the present study, we observed the effects of Nrf2 knockdown on cell proliferation, intracellular redox status and the functions of cellular signal transduction pathways. We found that Nrf2 deficiency led to a decrease in U251 cell proliferation and caused intracellular redox imbalance (decreased levels of GSH and increased levels of ROS). Moreover, Nrf2 depletion also resulted in the inhibition of AKT and ERK1/2 signaling. We further found that GME supplementation reversed the Nrf2 deficient-induced cell growth arrest and restored intracellular GSH levels. In addition, GME supplementation also restored AKT signaling

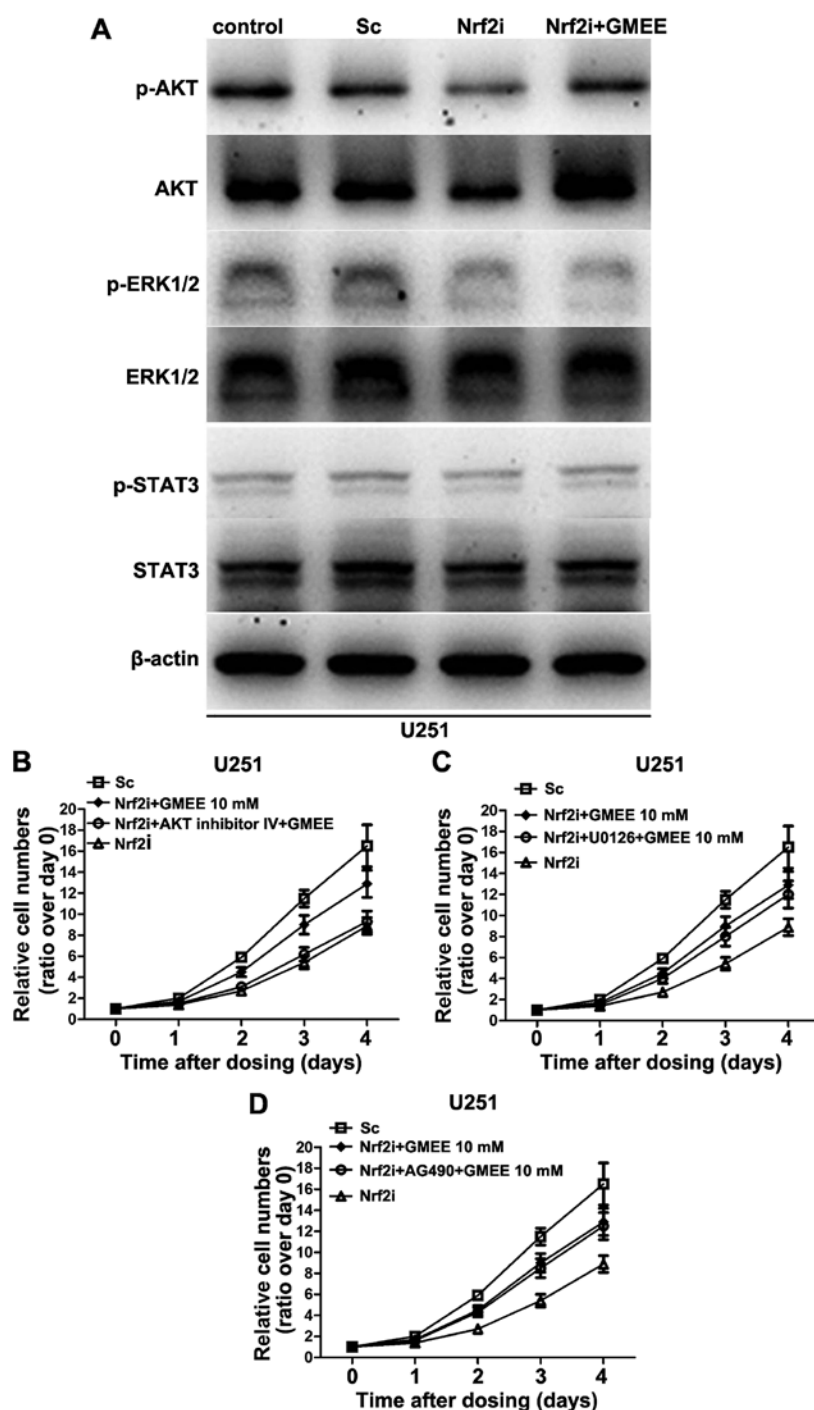


Figure 5. AKT kinase mediates the GMEE-induced Nrf2-deficient U251 cell proliferation. (A) Disruption of Nrf2 resulted in dysfunctional AKT and ERK1/2 signaling, while GMEE supplementation only restored AKT activation. Cells were grown in the absence or presence of 10 mM GMEE or 10 mM NAC for 4 days. On day 4 cell lysates from the non-treated (control), scrambled control (Sc) shRNA and Nrf2-shRNA (Nrf2i)-treated U251 cells were prepared and immunoblotted with antibodies specific for AKT, ERK1/2 and STAT3. β -actin was used as a loading control. (B-D) Inhibition of ERK1/2 and STAT3 had no significant effects on GMEE-induced Nrf2-deficient cell proliferation (C and D). In contrast, blocking AKT signaling with an AKT-specific inhibitor markedly blocked the Nrf2-deficient cell proliferation induced by GMEE (B). Nrf2-deficient U251 cells were incubated with either GMEE (10 mM) alone or respectively combined with the AKT inhibitor (AKT inhibitor IV, 5 μ M), ERK1/2 inhibitor (U0126, 10 μ M) and STAT3 inhibitor (AG490, 10 μ M) for 4 days. Cell viability was assessed every 24 h by CCK-8 assay.

but not ERK1/2 signaling, although they were both suppressed in the Nrf2-knockdown U251 cells. Notably, we unexpectedly found that supplementation of cells with antioxidant NAC appreciably decreased ROS levels, but it failed to counteract the phenotypic defects associated with cell proliferation. Finally, we provided evidence that the inhibition of ERK1/2 and STAT3 had no significant effect on GMEE-induced

Nrf2-deficient cell proliferation. In contrast, blocking AKT signaling with an AKT-specific inhibitor markedly blocked the Nrf2-deficient cell proliferation induced by GMEE, confirming that AKT signaling is important in GMEE-induced Nrf2-deficient cell proliferation. In the present study, we first revealed that Nrf2 knockdown induced inhibition of proliferation possibly by GSH depletion and consequent AKT

signaling inhibition, a new perspective by which to understand the role of Nrf2 activation in cancer pathogenesis.

Of particular note is our observation that supplementation of Nrf2-knockdown cells with the antioxidant *N*-acetylcysteine (NAC), a precursor of GSH, markedly lowered ROS levels but failed to induce cell proliferation. Several studies have shown that NAC attenuates or provides protection against pro-oxidant stimuli both *in vitro* and *in vivo* (24,25). Consistent with these results, our experiments also demonstrated that NAC could markedly squelch ROS levels, but it was unable to restore the decreased intracellular GSH levels that were present in Nrf2-knockdown U251 cells. The inability of NAC to restore the decreased GSH levels in Nrf2-knockdown cells is not surprising, since these cells expressed decreased levels of the γ -glutamyl cysteinyl ligase (GCL) components GCLC and glutathione reductase (GR), whose activities are essential for GSH generation from its precursor NAC (26-28). Although the sources contributing to the high levels of ROS in Nrf2-knockdown cells remain to be investigated, it is likely that decreased levels of GSH may lead to an inefficient cellular detoxification of endogenous ROS levels generated by mitochondria constitutively in Nrf2-silenced cells. In addition, it is possible that Nrf2 deficiency may result in dysregulation of ROS generating enzymes, such as Nox/Duox, Rac or RAS, thereby contributing to increased levels of ROS. Based on the aforementioned results, we directly treated the Nrf2-deficient cells with GMEE (a membrane-permeable derivative of GSH that can supplement cellular GSH levels) in culture medium. As expected, in addition to squelching the high levels of ROS, GMEE supplementation reversed Nrf2 deficiency-induced cell growth arrest and restored the intracellular GSH levels. Thus, it is likely that Nrf2-knockdown cells may only inefficiently convert NAC to GSH, a process that may otherwise cause the intracellular GSH to rise to levels that are adequate for cell proliferation.

To further define the mechanisms contributing to the growth arrest caused by Nrf2 deficiency, we assessed the status of the cellular signaling transduction pathways, since we reasoned that decreased GSH levels in Nrf2-knockdown cells may cause a dysregulation of signal transduction pathways that are related to cell proliferation. This notion is based on recent studies that have suggested a role for protein glutathionylation, a reversible post-translational mechanism involved in the modulation of the activities of the redox-sensitive thiol proteins, particularly those involved in signal transduction and cell proliferation (29). In the present study, we found that suppression of Nrf2 expression could lead to AKT and ERK1/2 pathway inhibition (the phosphorylation status of STAT3 exhibited no significant alterations), which are both known to be important in cell proliferation. However, treatment of Nrf2-knockdown cells with GMEE revealed that exogenous supplementation with GMEE could only restore AKT signaling but not ERK1/2 signaling, even though they were both suppressed in the Nrf2-knockdown U251 cells. Knockdown of Nrf2 in U251 cells caused the dysfunction of a series of signal transduction pathways, but GMEE supplementation only restored them in part, not completely. These observations could be used to explain the results obtained as indicated in Fig 4 which revealed that exogenous supplementation of Nrf2-deficient cells with GMEE did not

induce cell proliferation to the same extent as was observed in the counterparts.

To further confirm these results, we finally provided evidence that the inhibition of ERK1/2 and STAT3 had no significant effects on GMEE-induced Nrf2-deficient cell proliferation, whereas, blocking AKT signaling with an AKT-specific inhibitor markedly blocked the Nrf2-deficient cell proliferation induced by GMEE, confirming that AKT signaling is important in GMEE-induced Nrf2-deficient cell proliferation. Our data are consistent with a previous study (30) in which Reddy *et al* found that Nrf2 deficiency impaired AKT signaling in primary alveolar epithelial cultures isolated from Nrf2^{-/-} mice. However, this research group did not point out the precise crosstalk mechanism between AKT signaling and Nrf2. In the present study, we provide evidence of the crosstalk between these two pathways suggesting that protein (AKT kinase) de-glutathionylation (induced by intracellular decreased GSH levels) may be involved in the AKT inhibition resulting from Nrf2 disruption.

Emerging studies have pointed out that Nrf2 and its downstream target genes play a potential role in tumorigenesis, but the detailed underlying mechanism of the biological effects of Nrf2 activation in cancer remain elusive. In the present study, we revealed the decrease in Nrf2-impaired GBM cell proliferation via GSH depletion and consequent AKT signaling inhibition. For the first time the underlying mechanism of Nrf2 in cancer cell proliferation from the perspective of cellular metabolism, which enhances our comprehension of the role of Nrf2 in tumorigenesis was elucidated. More significantly, knowledge of the association of Nrf2 and cellular metabolism-related signaling pathways in cancer cells, may provide new strategies for glioma clinical intervention, particularly in the absence of effective Nrf2 inhibitors.

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