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Original Article

NDRG2 inhibits pyruvate carboxylase-mediated anaplerosis and combines with glutamine blockade to inhibit the proliferation of glioma cells

Jiancai Wang[1,2*], Xiang Sun[3*], Jiayuan Wang[1,4], Kun Zhang[1,5], Yiyi Yuan[1], Yan Guo[1], Libo Yao[1], Xia Li[1], Lan Shen[1]

*[1]The State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, The Fourth Military Medical University, Xi'an 710032, Shaanxi, China; [2]Department of Neurosurgery, PLA 982 Hospital, Tangshan 063099, Hebei, China; [3]Department of Special Diagnosis, School of Stomatology, The Fourth Military Medical University, Xi'an 710032, Shaanxi, China; [4]Department of Pathogenic Biology, Medical College, Yan'an University, Yan'an 716000, Shaanxi, China; [5]Department of Medical Genetics, Medical College, Yan'an University, Yan'an 716000, Shaanxi, China. *Equal contributors.*

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Abstract: Due to the rapid proliferation, cancer cells have increased anabolic biosynthesis, which requires anaplerosis to replenish precursor intermediates. The major anaplerotic sources are pyruvate and glutamine, which require the catalysis of pyruvate carboxylase (PC) and glutaminase (GLS) respectively. In GLS-suppressed cancer cells, the PC-mediated pathway for anaplerosis is crucial to maintain cell growth and proliferation. Here, we investigated the regulatory role and molecular mechanism of N-myc downstream-regulated gene 2 (NDRG2) in PC and PC-mediated anaplerosis. NDRG2 interacted with PC and induced the degradation of PC in glutamine-deprived cells. NDRG2 also inhibited the activity of PC and PC-mediated anaplerosis. As a result, NDRG2 significantly inhibited the malignant growth and proliferation of glioma cells in combination with a glutamine antagonist. In addition, NDRG2 more significantly inhibited the protein level of PC in isocitrate dehydrogenase 1 (R132H)-mutant glioma cells than in wild-type glioma cells. These findings indicate that the molecular mechanism of NDRG2 inhibits PC-mediated anaplerosis and collaborates with glutamine antagonist to inhibit the malignant proliferation of glioma cells, thus providing a theoretical and experimental basis for targeting anaplerosis in glioma therapy.

Keywords: NDRG2, anaplerosis, puruvate carboxylase, glutamine, glioma

Introduction

Malignant growth and proliferation of cancer cells require large quantities of bioenergy and biomaterials [1]. Many biosynthetic precursors for proteins, lipids and nucleotides are generated from the tricarboxylic acid (TCA) cycle. When the intermediates are removed from the TCA cycle, additional metabolic pathways are necessary to supply oxaloacetate (OAA) and TCA cycle intermediates. These OAA-generating metabolic pathways are named anaplerosis, which is used to replenish precursor intermediates [2, 3]. The major anaplerotic resources are pyruvate and glutamine, which require the enzymatic activity of pyruvate carboxylase (PC) and glutaminase (GLS), respectively [4]. When glutamine is deprived, PC-mediated anaplerosis is important for the growth and proliferation of cancer cells.

Gliomas are the most common and the most malignant brain tumors. The World Health Organization divides gliomas into four grades [5]. Grade I gliomas are benign, grades II and III are diffuse gliomas that are invasive and can progress into grade IV, which are also named glioblastomas [6]. Somatic mutations in the isocitrate dehydrogenase 1 (IDH1) gene occur at a very high frequency in grade II/III gliomas and secondary glioblastomas, and IDH1 mutations seem to be a prognostic factor for survival in glioma patients [7, 8]. IDH1 mutations, which commonly occur at the R132 residue in the active site, lead to the production of the oncometabolite 2HG and tumorigenesis [9]. Furthermore, IDH1 mutations elicit additional metabolic changes, especially in reprogramming of pyruvate metabolism [9]. Pyruvate dehydrogenase (PDH) activity is decreased while pyruvate carboxylase (PC) activity and expression are increased in IDH1-mutated glioma cells, which results in the enhancement of PC-mediated TCA anaplerosis [9, 10]. PC and PC-mediated TCA anaplerosis may be potential targets in IDH1-mutated gliomas.

N-myc downstream-regulated gene 2 (NDRG2), a member of the NDRG family, was firstly discovered by our laboratory using subtractive hybridization [11]. Our previous studies have demonstrated that NDRG2 is expressed widely in normal tissues [12], and is decreased in glioblastoma and other types of tumor tissues [11, 13-16]. NDRG2 can inhibit the malignant growth and proliferation of tumor cells [11, 17, 18], and is therefore considered as a tumor suppressor gene [19, 20]. Moreover, NDRG2 suppresses tumor metabolic reprogramming. Our previous research showed that NDRG2 inhibited glycolysis and glutaminolysis by coordinately targeting glucose and glutamine transporters, multiple catalytic enzymes involved in glycolysis and glutaminolysis of cancer cells [1]. NDRG2 also inhibited the activation of fatty acid oxidation and suppressed the glucose deprivation-induced AMPK/ACC pathway activation in cancer cells [21]. As a tumor suppressor gene, NDRG2 plays important roles in tumor metabolic reprogramming.

In this study, we aimed to investigate the regulatory role of NDRG2 in pyruvate carboxylase and pyruvate carboxylase-mediated anaplerosis. We found that NDRG2 interacted with PC and induced the degradation of PC, thus inhibiting the activity of PC and PC-mediated anaplerosis. Moreover, NDRG2 combined with a GLS inhibitor significantly inhibited the malignant growth and proliferation of glioma cells. Our findings may provide potential molecular targets for glioma therapy.

Materials and methods

Cell cultures and reagents

The human glioma cell lines U251, T98G, IDH1 (R132H) U87, IDH1 (WT) U87 and HEK293T cells were purchased from ATCC and used in the present study. Cells were maintained in the medium recommended and supplemented with 10% FBS in a 37°C and 5% CO_2 incubator. Recombinant lentiviral vectors were constructed with an ViraPower™ Lentiviral System (Invitrogen, Carlsbad, CA) in our laboratory [1]. The lentiviral vectors pLenti6-mCherry/NDRG2, PAX2 and PMD2G were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Glioma cells were infected with viral medium from HEK293T cells 48 h after transfection. Cycloheximide (CHX), which blocks the elongation phase of eukaryotic translation and eukaryote protein synthesis, is used to determine the half-life of PC protein. MG-132, which blocks the proteolytic activity of the 26S proteasome complex and acts as a proteasome inhibitor, is used to inhibit PC protein degradation via ubiquitin-proteasome system. L-DON (L-6-Diazo-5-oxonorleucine), which is a glutamine analog and glutaminase inhibitor, is used to mimic the effects of glutamine deprivation *in vivo* tumorigenicity model. CHX, MG-132 and L-DON were purchased from Selleckchem (Houston, TX, USA).

MTT assay

Infected cells were seeded in 96-well plates in triplicate at a starting density of 1×10^4 cells/well and cultured in the recommended medium without glutamine. Treated cells were washed and incubated with 100 μg/ml tetrazolium salt (MTT, Sigma Aldrich, St. Louis, MO) at 37°C for 4 h. The supernatant was removed, and 150 μl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance (OD) of the reaction solution at 490 nm was recorded.

Colony formation assay

Infected cells were seeded into 60-mm dishes at a density of 400 cells per dish. The cells were grown for 2 weeks in culture medium without glutamine. Then, the colonies were fixed and stained with crystal violet.

Western blotting

For Western blotting analysis, total protein was prepared from human glioma cell lines and HEK293T cell line. Immunoblotting was per-

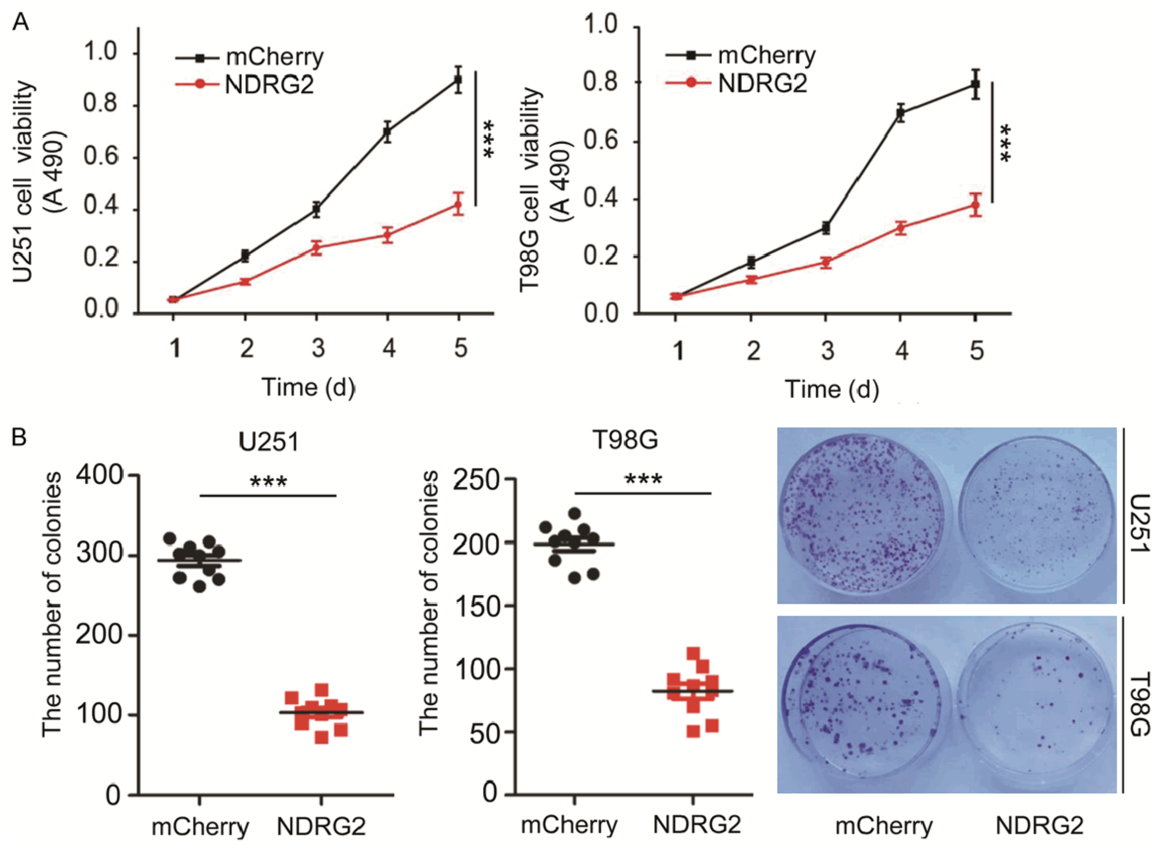

Figure 1. NDRG2 inhibits the proliferation of glutamine-deprived glioma cells. A. U251 and T98G cells were infected with lentivirus containing NDRG2 or mCherry, and cell viability was measured with MTT assays. B. Equal numbers of NDRG2-overexpressing U251 (T98G) cells and control cells were seeded onto 60 mm diameter dishes. After 14 days, the cells were fixed and stained with crystal violet. All data shown are the mean ± SD of three independent experiments. ****P*<0.001.

formed according to standard procedures with monoclonal rabbit anti-PC antibody (1:2000, Abcam, ab126707), monoclonal mouse anti-NDRG2 antibody (1:1000, Abnova H00057-447-M03), monoclonal mouse anti-Flag antibody (1:1000, Sigma, F3165), monoclonal mouse anti-Myc tag antibody (1:1000, Abcam, ab32), monoclonal mouse anti-HA tag antibody (1:1000, Abcam, ab18181), monoclonal mouse anti-IDH1 (R132H) antibody (1:200, Sigma, ASB4200548), monoclonal mouse anti-α-tubulin antibody (1:1000, Boster, M03989-2) and monoclonal rabbit anti-β-actin antibody (1:1000, Boster, BM3873).

Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA), and then complementary DNA (cDNA) was synthesized using AMV reverse transcriptase (Promega, Mannheim, Germany) according to the manufacturer's instructions. cDNA was used as a template for quantitative real-time PCR using an ABI Prism 7500 real-time PCR instrument (BioRad, Hercules, CA). The primers used for real-time quantitative PCR are listed in the Supplementary Table 1.

Vector construction

The construction scheme of the expression vector used for tandem affinity purification (TAP) is shown in **Figure 2A**. For construction of the expression vector, the coding genes of NDRG2 were amplified by PCR and confirmed by DNA sequencing, and then the target genes were ligated with a fusion expression vector containing S-tag, Flag and streptavidin-binging peptide (SBP). The primers used for PCR are listed in the Supplementary Table 2.

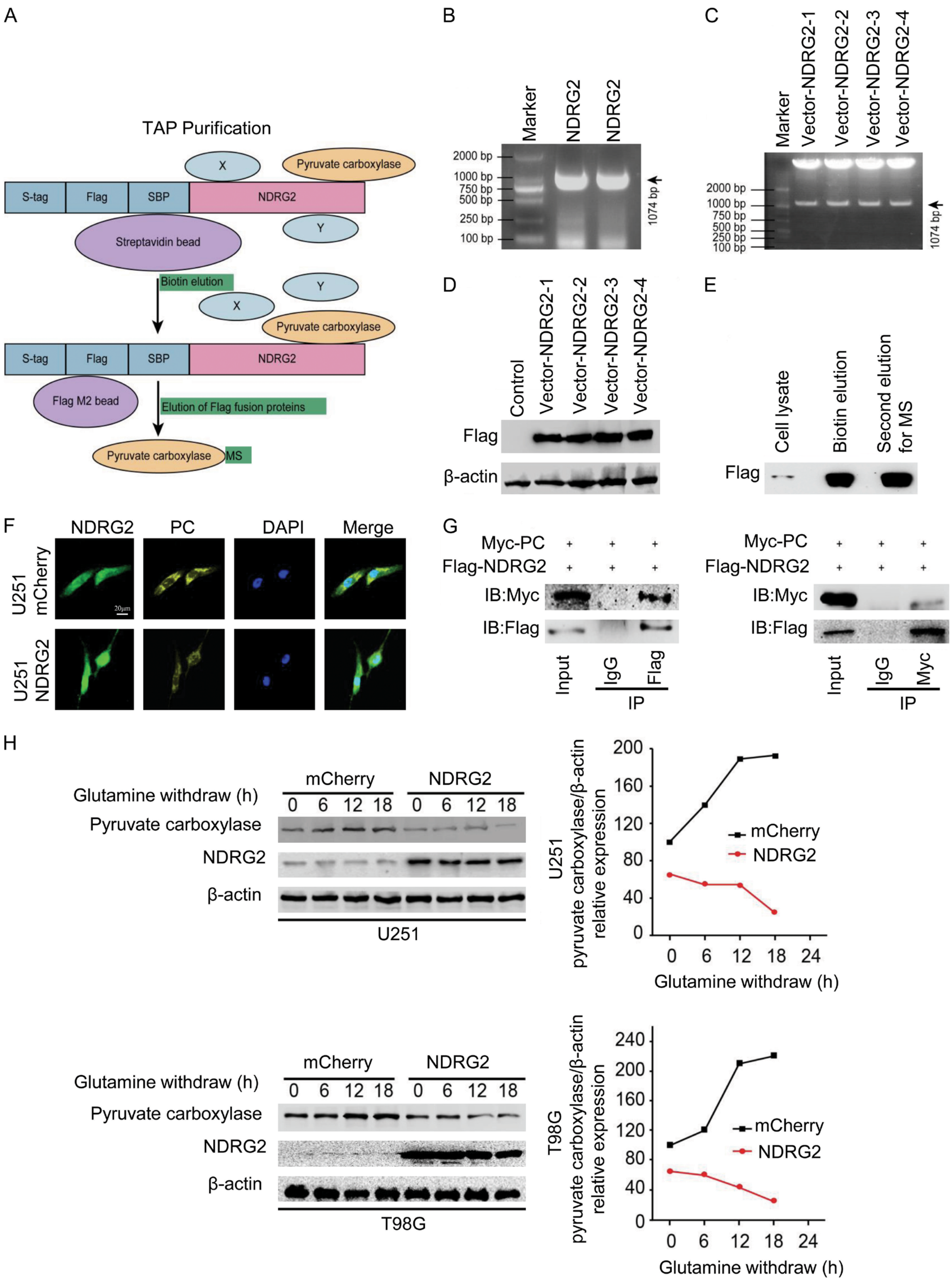

Figure 2. NDRG2 interacts with pyruvate carboxylase and blocks the upregulation of pyruvate carboxylase expression in glutamine-deprived glioma cells. A. Graphic illustration of the tandem affinity and purification procedure for mass spectrometry (TAP-MS). B. PCR amplification of the NDRG2 gene from human astrocyte cDNA. C. Digestion identification of recombinant plasmid S protein-Flag-Streptavidin binding protein tag-NDRG2 (SFB-NDRG2) with restriction enzyme by agarose gel electrophoresis. D. HEK293T cells were transfected with the recombinant plasmid SFB-NDRG2, and Flag tag expression was detected by immunoblotting using an anti-Flag antibody. E. Purified protein complexes for mass spectrometry were detected by immunoblotting using an anti-Flag antibody. F. Subcellular

localization of NDRG2 and pyruvate carboxylase in U251 cells. Immunocytochemical staining of U251 cells was performed using NDRG2 or PC antibody, and visualized using FITC- or Cy3-conjugated secondary antibody to show the localization of NDRG2 (green) and PC (yellow). DAPI staining (blue) reveals the cell nuclei. Scale bar, 20 μm. G. Western blot analysis of ectopically expressed Flag-NDRG2 and Myc-PC reciprocally immunoprecipitated by anti-Myc or anti-Flag in HEK293T cells. H. U251 and T98G cells were infected with NDRG2 or mCherry lentivirus for 48 hours, and then cultured in DMEM medium without glutamine for the indicated periods. The protein levels of pyruvate carboxylase and NDRG2 at different times were detected by Western blotting analysis. The relative protein levels of pyruvate carboxylase to β-actin were quantified by densitometry.

Tandem affinity purification

HEK293T cells were transfected with an SFB-tagged NDRG2 or empty vector. Twenty-four hours post-transfection, the cells were lysed in NETN buffer (20 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40, 50 mmol/L b-glycerophosphate, 10 mmol/L NaF, and 1 mg/mL pepstatin A) at 4°C for 3 hours. The supernatant was collected for incubation with Streptavidin-Sepharose beads (GE Healthcare Sciences) at 4°C overnight. The next day, the beads were washed with NETN buffer five times and then eluted with 2 mmol/L biotin (Sigma) for 1 hour at 4°C twice. The elution products were incubated with S-protein agarose beads (Novagen) at 4°C overnight, and after three washes, the products bound to S-protein agarose beads were subjected to SDS-PAGE and analyzed by mass spectrometry (MS).

Coimmunoprecipitation

Cells were harvested and lysed in IP buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1% protease inhibitor cocktails) on ice for more than 15 minutes. Cell lysates were centrifuged for 10 minutes at 13,000 rpm at 4°C, and the supernatant was transferred to a new tube. The supernatant was incubated with primary antibodies against Myc or Flag and protein A/G agarose beads (Thermo Fisher Scientific) with gentle rocking at 4°C overnight. The next day, the pellet was washed six times with IP buffer on ice and then subjected to Western blotting analysis.

Immunohistochemistry

Glioma specimens were histologically diagnosed, and glioma tissue microarrays (TMAs) were produced by the Department of Pathology, Xijing Hospital, Fourth Military Medical University. Tissue microarray (TMA) staining was performed using standard immunohistochemistry procedures. The slides were incubated overnight with primary antibodies against NDRG2 (1:500, Abnova, H00057447-M03) or PC (1:500, Abcam, ab229267). Staining intensity was scored in a blinded fashion: 1 = weak staining at ×100 magnification but little or no staining at ×40 magnification; 2 = medium staining at ×40 magnification; 3 = strong staining at ×40 magnification. The final staining index was calculated using the following formula: staining intensity × percentage.

Tumor tissues from nude mice were collected on day 28, excised and fixed with 4% formalin, and embedded in paraffin. For immunohistochemistry, 5 μm-thick tissue sections were cut, dewaxed in xylene, and rehydrated. For Ki67 staining, the slides were incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) at room temperature for 1 h for blocking and then stained with primary antibodies against NDRG2 (1:500, Abnova, H00-057447-M03), PC (1:500, Abcam, ab229267) or Ki-67 (1:1000, Invitrogen, PA5-19462) at room temperature for 4 h. The slides were subsequently washed three times with PBS to remove excess primary antibody and then incubated with anti-mouse HRP-conjugated IgG (1:500, Boster, BM3895) for 1 h at room temperature. Finally, the slides were washed three times, incubated with diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate (Sigma) and covered with glass cover slips. The staining results were observed with a bright field microscope.

For NDRG2 and PC subcellular localization, the cells were fixed in a freshly prepared solution of 4% paraformaldehyde, rinsed, and permeabilized with 0.1% Triton X-100 in PBS. Permeabilized cells were then incubated with horse serum in PBS to block nonspecific binding. After thorough rinsing with PBS, the cells were incubated overnight with NDRG2 or PC antibody, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody or Cy3-conjugated anti-mouse antibody. Dual-color detection was performed by confocal laser scanning microscopy after treatment with 4',6-diamidino-2-phenylindole (DAPI) for 10 min to label nuclear DNA. Specimens were examined with a FV300 laser scanning microscope (Olympus, Tokyo, Japan).

Pyruvate carboxylase activity

Treated cells were seeded on 6-well plates at a density of 1×10^6 cells per well and the culture medium was changed to low glucose DMEM without phenol red (Thermo Fisher Scientific). The activity of pyruvate carboxylase in the culture medium was measured after incubation of cells for 24 h with a pyruvate carboxylase activity assay kit (Jiancheng Bioengineering, China). The activity of pyruvate carboxylase was normalized to the cell numbers. The cell numbers were calculated and analyzed using a Cellometer Mini bright field automated cell counter (Nexcelom Bioscience, USA).

Tracer studies in cell cultures

U251 cells transduced with NDRG2 or mCherry were incubated in DMEM with 10 mM ^{13}C-glucose and in the absence of glutamine for 18 hours, quenched in cold acetonitrile, and extracted in acetonitrile/water/chloroform (v/v 2:1.5:1). Metabolite fractions from cells were analyzed by Gas chromatography-mass spectrometer (GC-MS) as previously reported [22].

In vivo tumorigenicity assay

The animal study and experimental protocols were approved by the Institutional Laboratory Animal Center at the Fourth Military Medical University. The animals were maintained and handled in accordance with the Guidelines for Accommodation and Care of Animals. JC Wang has a license for animal experiments. All mice were housed under standard conditions of a 12-hour light/dark cycle and access to food and water ad libitum. To generate tumors *in vivo*, 1×10^6 U251 cells in PBS were injected subcutaneously into the franks of nude mice (6 mice in each group, male BALB/C-nu/nu, 18-20 grams, 6 weeks old). U251 cells expressing the mCherry control were injected into the left flank, and U251 cells expressing NDRG2 were injected into the right flank. When the tumor size reached an average of 80 mm^3, the mice were treated with 15 mg/kg L-DON or PBS 3 times per week. The tumor size was measured every other day, and tumor volume was calculated using a standard formula: tumor volume (mm^3) = width (mm^2) × length (mm) × 0.5. After 21 days of drug treatment, the mice from each group were sacrificed. The final weight of tumor mass was measured and the tumor tissues were harvested for immunohistochemistry staining.

Statistical analysis

Statistical analyses were conducted using SPSS 16.0 (IBM, New York) and Origin 6.0 (Microcal Software, Inc., Northampton). All results are presented as the mean ± SD. All experiments were performed in triplicate. Student's t-test was used to compare the differences between two groups. Pearson product-moment correlation was used to calculate the correlation between NDRG2 and PC staining index in IDH1 wild-type and IDH1-R132H mutant glioma TMAs. Differences between groups were considered significant when $P<0.05$.

Results

NDRG2 inhibits the proliferation of glutamine-deprived glioma cells

Tumor suppressor gene NDRG2 is highly expressed in glial cells and significantly down-regulated in glioma tissues and cells [11]. To investigate the effect of NDRG2 gain-of-function on glioma cells, we initially examined the growth and proliferation of NDRG2-overexpressing glioma cells in the glutamine-depleted culture medium by MTT and colony formation assays. Interestingly, ectopic NDRG2 expression markedly diminished the growth and proliferation of U251 and T98G cells in the glutamine-depleted culture conditions (**Figure 1A**, **1B**). NDRG2 maybe block the necessary nutritional support for the proliferation of glutamine-deprived glioma cells.

NDRG2 interacts with pyruvate carboxylase and blocks the upregulation of pyruvate carboxylase expression in glutamine-deprived glioma cells

To explore the molecular mechanism of NDRG2 inhibiting glioma proliferation, tandem affinity purification-mass spectrometry (TAP-MS) assays were used to search for the interacting proteins of NDRG2. We first constructed a NDRG2 mammalian expression vector, which contains S, streptavidin-binding-peptide, and Flag tags (**Figure 2A**). The cDNA coding fragment of NDRG2 was amplified from human brain mRNA by RT-PCR (**Figure 2B**) and cloned into the pMD18-T vector to analyze the nucleotide sequence. The results showed that the DNA sequence of the cloned human NDRG2 domain was consistent with that reported previously [11]. The NDRG2 cDNA was subcloned into the expression vector, and the recombinant plasmid was digested with restriction enzymes (**Figure 2C**). The recombinant plasmid and the backbone vector were individually transfected into HEK293T cells, and the expression of NDRG2 fusion protein was detected by Western blotting (**Figure 2D**). Then, HEK293T cell extracts were prepared for tandem affinity protein purification to obtain the NDRG2 protein complex, which was then detected by mass spectrometry. Multiple binding partners of NDRG2, including pyruvate carboxylase, were identified by mass spectrum (**Figure 2E**; Supplementary Table 3).

NDRG2 is mainly located in the cytoplasm of astrocytes, and pyruvate carboxylase is mainly located in the mitochondria [12, 13, 23]. Therefore, we detected the colocalization and interaction of NDRG2 and pyruvate carboxylase in glioma cells. Our indirect immunofluorescence results showed that NDRG2 and pyruvate carboxylase were well colocalized in U251 cells (**Figure 2F**, Supplementary Figure 1). Moreover, the interaction between ectopically expressed Flag-NDRG2 and Myc-PC in HEK293T cells was confirmed by reciprocal coimmunoprecipitation (co-IP) assays (**Figure 2G**).

Under glutamine deprivation, PC-mediated anaplerosis is important for the growth and proliferation of cancer cells. Therefore, we investigated the regulatory effect of NDRG2 on the expression of pyruvate carboxylase in the absence of glutamine metabolism. We observed the transcription and protein levels of pyruvate carboxylase in glutamine-depleted glioma cells with ectopic NDRG2 expression. Western blotting analysis showed that the expression of pyruvate carboxylase increased under glutamine deprivation in the glioma cell lines U251 and T98G. However, the expression of pyruvate carboxylase decreased slightly in the NDRG2 -overexpressing U251 and T98G cells (**Figure 2H**). Interestingly, the mRNA level of pyruvate carboxylase increased under glutamine deprivation in glioma cells (Supplementary Figure 2). NDRG2 blocked the upregulation of pyruvate carboxylase expression induced by glutamine deprivation in glioma cells at the protein level. These results suggest that pyruvate carboxylase may be regulated post-translationally by NDRG2 in glutamine-deprived cells.

NDRG2 induces the ubiquitination and degradation of pyruvate carboxylase under glutamine deprivation

As NDRG2 blocked the upregulation of pyruvate carboxylase expression induced by glutamine deprivation only at the protein level, we hypothesized that NDRG2 might regulate the protein stability and degradation of pyruvate carboxylase. To test this hypothesis, we infected the glioma cell lines U251 and T98G with lentivirus containing NDRG2 or mCherry, and then treated them with the protein synthesis inhibitor CHX under conditions of glutamine deprivation. The results of immunoblot analysis showed that the protein levels of pyruvate carboxylase decreased more significantly in the NDRG2-overexpressing glioma cells treated with cycloheximide, than in the control cells (**Figure 3A**). The degradation of pyruvate carboxylase was significantly accelerated by NDRG2 overexpression in the presence of CHX, suggesting that NDRG2 could promote the protein degradation of pyruvate carboxylase.

Ubiquitinated modification of proteins is usually involved in proteasome degradation [24]. We first examined whether the protein degradation of pyruvate carboxylase depends on the proteasome. As shown in **Figure 3B**, ectopic NDRG2 inhibited the protein level of pyruvate carboxylase, but the proteasome inhibitor MG-132 blocked the decrease of pyruvate carboxylase in the NDRG2-overexpressing U251 and T98G glioma cells under conditions of glutamine deprivation. Second, we examined whether NDRG2 affects the ubiquitination of pyruvate carboxylase. As shown in **Figure 3C**, overexpression of NDRG2 significantly promoted the ubiquitination of pyruvate carboxylase under conditions of glutamine deprivation. Therefore,

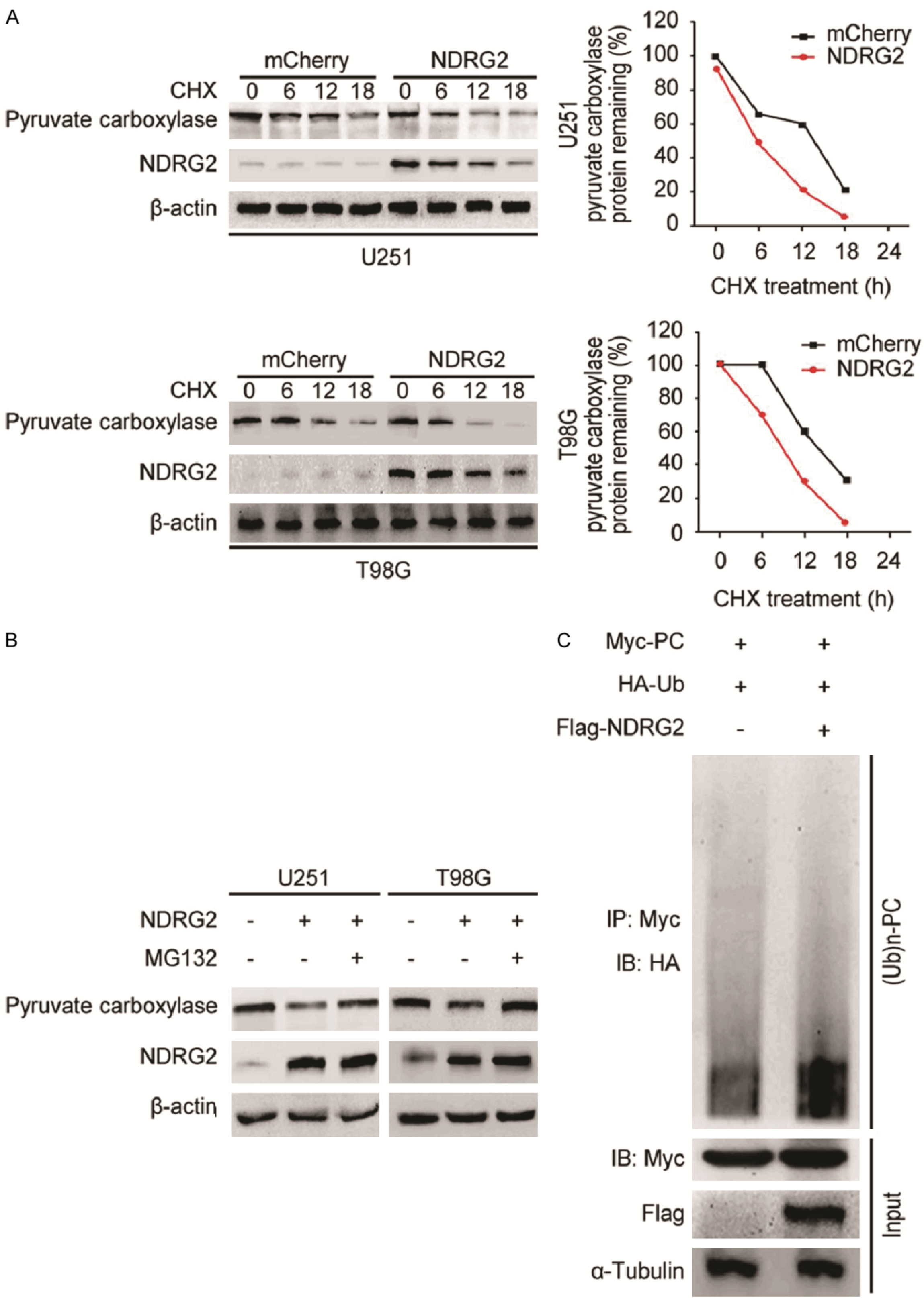

Figure 3. NDRG2 induces the degradation of pyruvate carboxylase upon glutamine deprivation. A. U251 and T98G cells were infected with NDRG2 or mCherry lentivirus for 48 hours, cultured in DMEM medium without glutamine and treated with 50 μM cycloheximide for the indicated periods. The protein levels of pyruvate carboxylase and

NDRG2 at different times were detected by Western blotting analysis. The relative protein levels of pyruvate carboxylase to β-actin were quantified by densitometry. B. U251 and T98G cells infected with NDRG2 or mCherry lentivirus were cultured in DMEM medium without glutamine and treated with 20 μM MG-132 for 18 hours, and Western blotting analysis of the protein levels of pyruvate carboxylase and NDRG2 was performed. C. Western blotting analysis of the products of the *in vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids and cultured in DMEM medium without glutamine was performed.

NDRG2 induces the ubiquitination and degradation of pyruvate carboxylase upon glutamine depletion.

NDRG2 inhibits the activity of pyruvate carboxylase and glucose-dependent anaplerosis through pyruvate carboxylase in glioma cells

Pyruvate carboxylase mediates glucose-dependent anaplerosis [3], we characterized the regulatory effect of NDRG2 on pyruvate carboxylase activity and anaplerosis mediated by pyruvate carboxylase. Our results showed that NDRG2 inhibited the activity of PC in U251 and T98G glioma cells. In the NDRG2 overexpressing U251 and T98G glioma cells, the activity of pyruvate carboxylase decreased (**Figure 4A**). To verify the inhibitory effect of NDRG2 on PC-mediated anaplerosis in cells, we incubated U251 cells that were infected with lentivirus containing NDRG2 or mCherry in ^{13}C-glucose for 24 hours and measured the metabolites for PC-mediated anaplerosis by GC-MS. The levels of ^{13}C-oxaloacetate, ^{13}C-citrate, ^{13}C-malate and ^{13}C-succinate were reduced in the NDRG2 overexpressing U251 cells compared with the control cells (**Figure 4B**). Therefore, NDRG2 inhibited PC-mediated anaplerosis in glioma cells.

NDRG2 combined with a glutamine antagonist predominantly inhibits the proliferation of glioma cells

Pyruvate carboxylase-mediated anaplerosis is required for the growth and proliferation of glioma cells [3]. Accordingly, we detected the effect of NDRG2 combined with a glutamine antagonist on the growth and proliferation of glioma cells *in vivo*. We established xenograft tumors using U251 cells overexpressing either mCherry or NDRG2. Once tumors were established, mice were treated with the glutaminase inhibitor L-DON for 21 days (**Figure 5A**). Importantly, the mice injected with U251 cells expressing NDRG2 and treated with L-DON developed tumors more slowly that the mice in the other groups (**Figure 5B-D**). In the related tumor sections, the protein levels of the proliferation marker Ki-67 and PC decreased significantly after NDRG2 overexpression and L-DON treatment (**Figure 5E**). Thus, NDRG2 combined with a glutamine antagonist can inhibit the malignant growth and proliferation of glioma cells.

The expression of NDRG2 has an inverse association with pyruvate carboxylase in IDH1 (R132H)-mutant glioma cells

Pyruvate carboxylase serves as a major source of TCA anaplerosis, while glutamine is used for 2-hydrozyglutarate production in IDH1 mutant glioma cells [10]. To fully address the clinical relevance of NDRG2 and pyruvate carboxylase in IDH1-R132H mutant glioma patients, we detected the protein levels of NDRG2 and pyruvate carboxylase in IDH1 wild type and IDH1-R132H mutant glioma tissues from 54 patients. Immunohistochemical and statistical analyses showed that there was no significant association between NDRG2 and pyruvate carboxylase expression in the IDH1 wild-type glioma tissues, but there was an inverse association between NDRG2 and pyruvate carboxylase expression in the IDH1-R132H mutant glioma tissues (**Figure 6A**, **6B**). We found that the expression of pyruvate carboxylase was upregulated in the IDH1-R132H mutant U87 glioma cells. However, ectopic NDRG2 expression decreased pyruvate carboxylase expression levels in the IDH1-R132H mutant U87 glioma cells (**Figure 6C**, **6D**). NDRG2 may inhibit the protein level of pyruvate carboxylase in the IDH1 mutant glioma cells.

In summary, this study illustrates the regulatory role and molecular mechanism of the tumor suppressor NDRG2 in PC and PC-mediated anaplerosis in glioma cells. In glucose homeostasis glia cells, NDRG2 induces the degradation of partial PC via ubiquitin proteasome pathway, and thereby partly inhibiting PC-mediated anaplerosis upon glutamine deprivation in mitochondria. In glioma cells, NDRG2 loss enhanced PC and PC-mediated anaplerosis, which facilitated the malignant proliferation of glioma cells (**Figure 7**). In addition, PC expres-

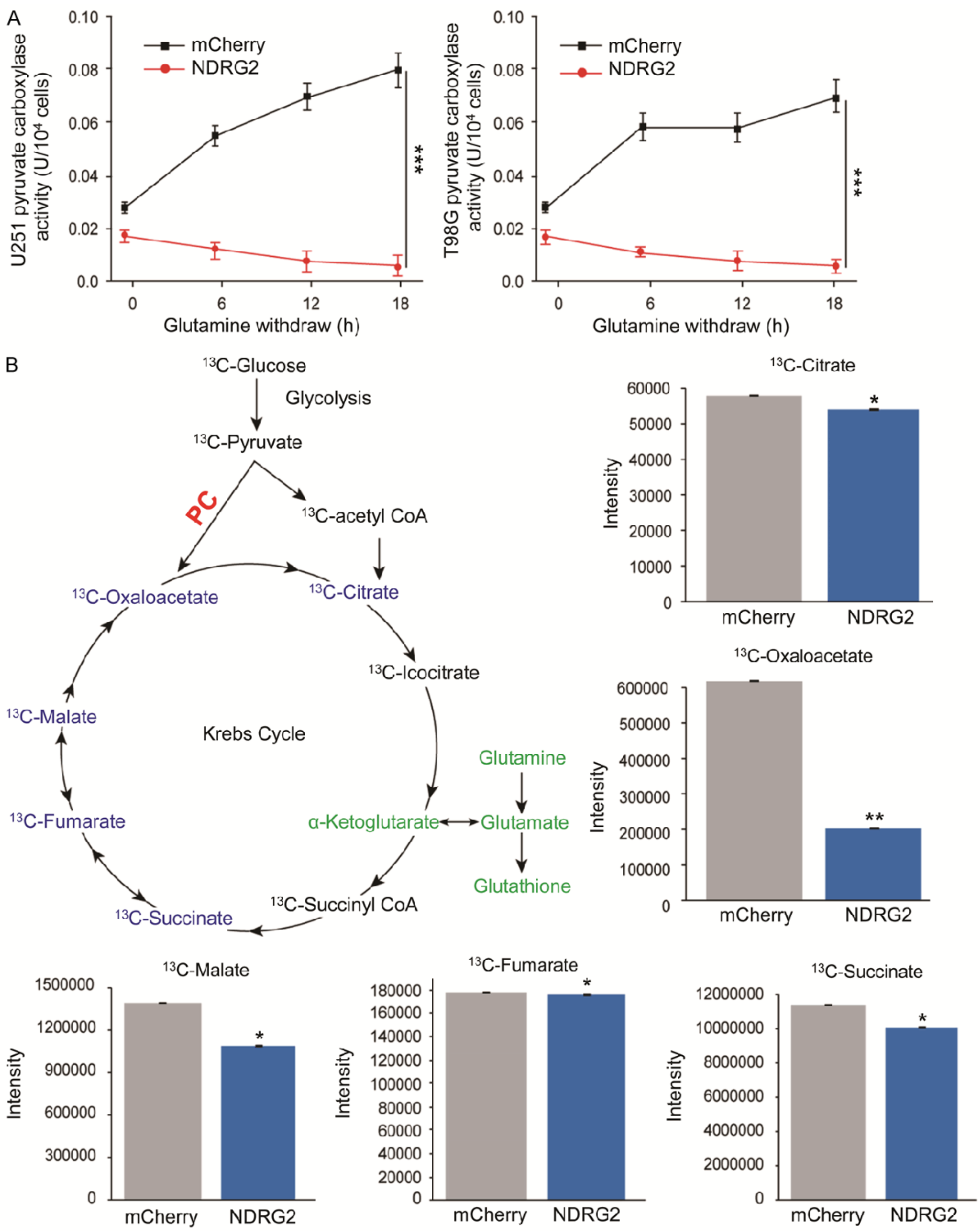

Figure 4. NDRG2 inhibits the activity of pyruvate carboxylase and glucose-dependent anaplerosis through pyruvate carboxylase in glioma cells. A. U251 and T98G cells were infected with lentivirus containing NDRG2 or mCherry for 48 hours and incubated with DMEM medium without glutamine for the indicated times. The activity of pyruvate carboxylase in U251 and T98G cells was detected by a pyruvate carboxylase activity assay kit according to the manufacturer's recommendation. Total enzyme activity was normalized to the cell number. B. U251 cells were infected with lentivirus containing NDRG2 or mCherry for 48 hours and incubated with DMEM medium containing ^{13}C-labeled glucose without glutamine for 18 hours. The ^{13}C-labeled organic acids in the TCA cycle were detected by GC-MS analysis. **P*<0.05, ***P*<0.01, ****P*<0.001.

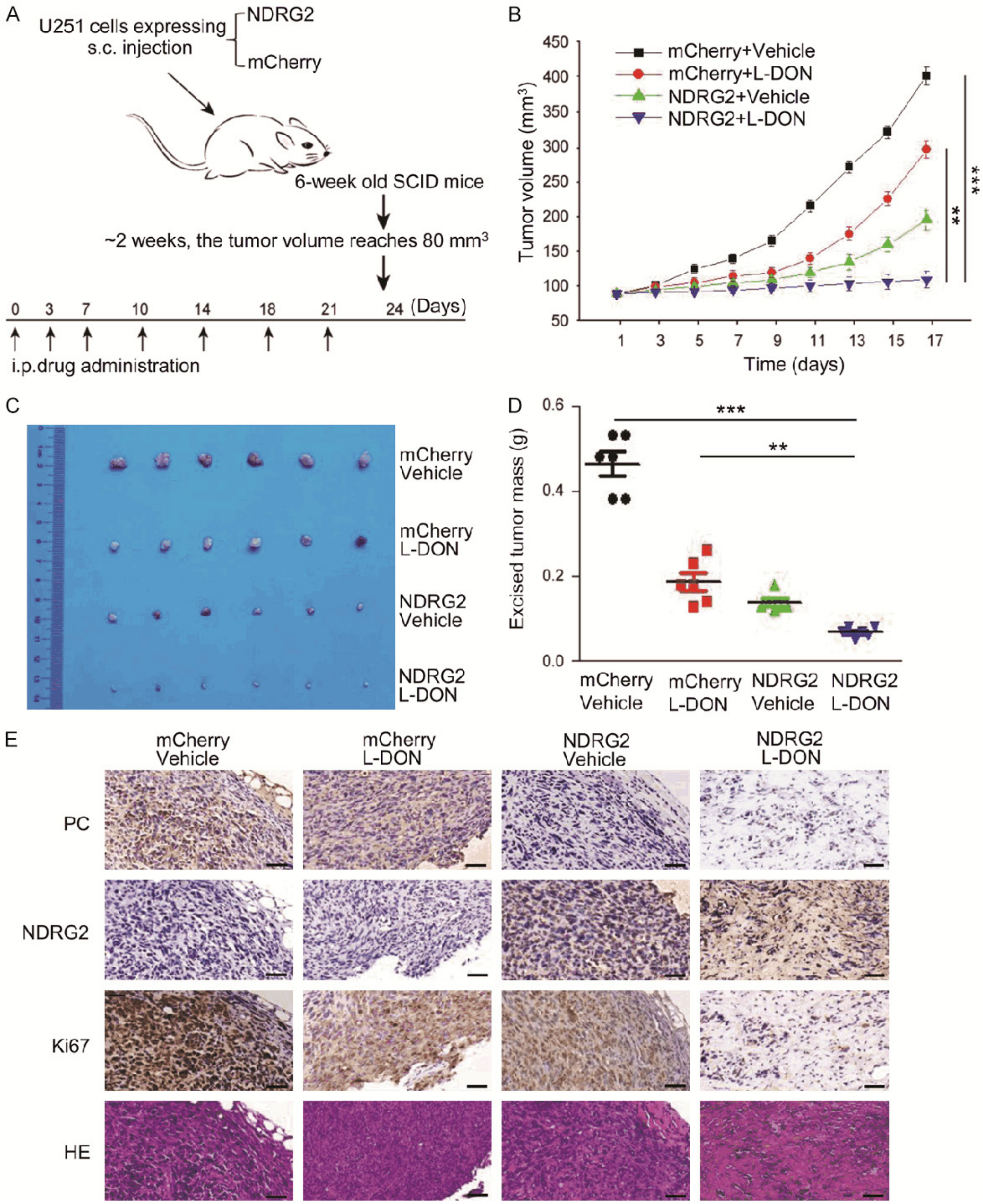

Figure 5. NDRG2 combined with glutamine antagonist to inhibit the proliferation of glioma cells *in vivo*. A. A schematic depicts the procedure of the establishment of NDRG2 overexpression xenograft models and inhibitor administration. Nude mice were injected subcutaneously with U251 cells that were infected with lentivirus containing NDRG2 or mCherry. Once the tumor size in nude mice of any group reached an average of 80 mm^3, all mice were treated with 15 mg/kg L-DON or PBS 3 times per week for three consecutive weeks. The control mice received saline alone. B. Tumor growth is shown. C. Representative tumor formation was photographed after the mice were sacrificed. D. Tumor weight was calculated at the end of the experiment. E. Immunochemical staining with antibodies specific for PC, NDRG2 and Ki67 in xenografts. Scale bar, 50 μm. All data shown are the mean ± SD of three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$.

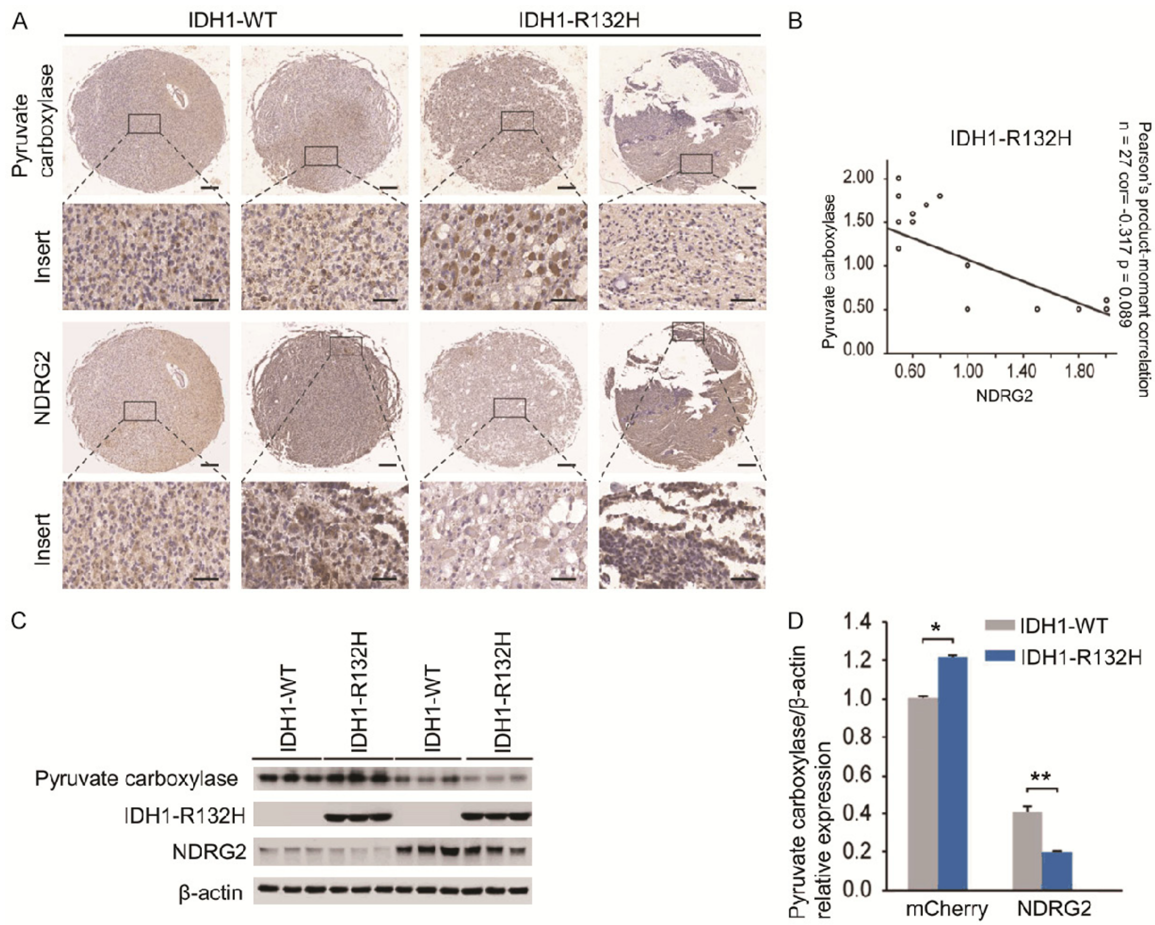

Figure 6. The expression of NDRG2 had an inverse association with pyruvate carboxylase in IDH1 (R132H)-mutant glioma cells. A. NDRG2 and pyruvate carboxylase immunostaining of tissue microarrays comprising IDH1 wild-type and IDH1 (R132H) glioma tissues with different differentiation states. Scale bar, 200 μm and 50 μm (magnification). B. Correlation analysis of the staining index for the expression of NDRG2 and PC proteins in the IDH1 wild-type glioma patient specimens (n = 27) and the IDH1-R132H mutant glioma patient specimens (n = 27). Pearson's product-moment correlation coefficients and the P values are also shown. C. NDRG2 and pyruvate carboxylase expression in the IDH1 wild-type and IDH1 (R132H) glioma U87 cells. D. The relative protein levels of pyruvate carboxylase to β-actin were quantified by densitometry. *P<0.05, **P<0.01.

sion was upregulated in the IDH1-R132H mutant U87 glioma cells compared with the IDH1 wild-type U87 glioma cells. NDRG2 inhibited the upregulation of PC expression in the IDH1 mutant U87 glioma cells. The inhibition of anaplerosis and proliferation may be one of the important reasons for NDRG2 as a tumor suppressor. Therefore, tumor suppressor NDRG2 is a potential therapeutic target for glioma.

Discussion

Tumor cells use glucose and glutamine to fuel anaplerosis and cell proliferation, which are catalyzed by pyruvate carboxylase and glutaminase respectively. Pyruvate carboxylase (PC) is the key anaplerotic enzyme that converts pyruvate to oxaloacetate in mitochondria, enabling the maintenance of other metabolic intermediates consumed by cataplerosis [25]. In glutamine-depleted tumor cells, the induction of a compensatory anaplerotic mechanism catalyzed by pyruvate carboxylase, allowed the cells to use glucose-derived pyruvate rather than glutamine for anaplerosis [3]. Glutamine blockade is widely used in cancer targeted therapy and immunotherapy, but resistance to glutamine blockade poses considerable challenges to cancer therapies. Our results suggest that tumor suppressor NDRG2 can enhance the sensitivity of cancer therapy targeting glutamine metabolism. Therefore, the use of combi-

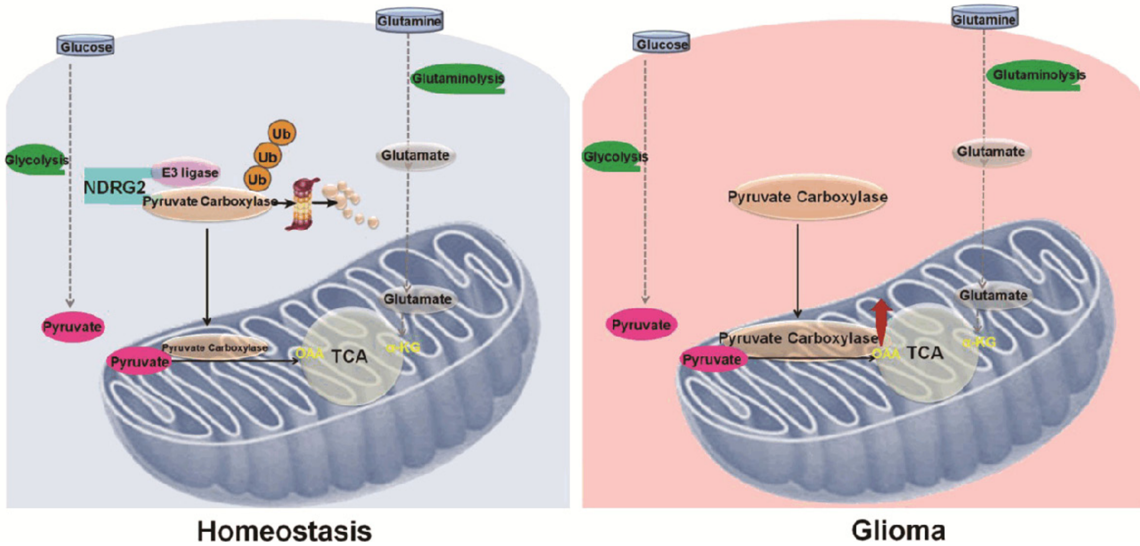

Figure 7. A schematic for the role of NDRG2 in regulating pyruvate carboxylase and pyruvate carboxylase-mediated anaplerosis in glioma cells. In normal glial cells, NDRG2 promotes the degradation of pyruvate carboxylase before PC translocates into the mitochondria (left). NDRG2 loss leads to an increase in PC- and PC-mediated anaplerosis under glutamine antagonism, and accelerates the tumorigenesis of glioma (right).

nation therapy is an effective strategy in the treatment of certain types of cancers [26].

Pyruvate carboxylase expression is upregulated in many types of cancer, including glioma. The promoter of the human PC gene contains the binding sites of several transcription factors, such as c-Myc, HIF1, SP1 and the vitamin D-responsive element (VDRE) [27]. These oncogenic transcription factors may contribute to the upregulation of PC gene expression in tumor cells. Glutamine blockade attenuates the production of biosynthesis and bioenergy, and therefore induces the inhibition of the mTOR signaling pathway and activation of AMPK [28, 29]. In addition, glutamine blockade leads to upregulation of the transcriptional factor c-Myc and activation of transcription factor 4 [30-32]. Our results showed that glutamine blockade induced the upregulation of pyruvate carboxylase at the transcriptional level, which is probably related to changes of transcription factors such as c-Myc or other signaling pathways. Clarifying the mechanism by which glutamine blockade induces the upregulation of pyruvate carboxylase expression could reveal new therapeutic targets for anaplerosis in cancer.

Pyruvate carboxylase is mainly located in mitochondria, which catalyzes the carboxylation of pyruvate to form oxaloacetate and maintain TCA cycle flux during robust biosynthesis [3]. The degradation of pyruvate carboxylase in mitochondria, similar to many enzymes in mitochondria, mainly occurs through the autophagic-lysosomal degradation pathway [33, 34]. Interestingly, our results showed that the tumor suppressor NDRG2 promoted the degradation of pyruvate carboxylase through the ubiquitin-proteasome degradation pathway in the cytoplasm, and maintained the remaining amount of pyruvate carboxylase entering the mitochondria. Although NDRG2 is not a E3 ligase, it still regulates the ubiquitination level of PC. Maybe NDRG2 acts as a scaffold protein and recruits E3 ligase to PC, therefore NDRG2 ubiquitinates PC indirectly. In addition, it was reported that NDRG2 enhanced the interaction of E3 ligase FBXO11 with Snail, and promoted Snail degradation by ubiquitination [35]. Whether E3 ligase FBXO11 is involved in the process of NDRG2 ubiquitinates PC needs further analysis and verification.

Tumor suppressor gene NDRG2, which is decreased in tumor cells, can inhibit tumor metabolic reprogramming and malignant proliferation. Our results showed that ectopic NDRG2 interacted with pyruvate carboxylase and induced the degradation of pyruvate carboxylase via the ubiquitin proteasome pathway. Moreover, NDRG2 inhibited PC-mediated anaplerosis and glioma cell proliferation upon glutamine deprivation. In addition, NDRG2 cooperated with a glutamine antagonist to suppress the malignant growth and proliferation of glioma cells. It is possible that the tumor suppressor gene NDRG2 induces sensitivity to glutamine antagonism-targeted cancer therapies.

Gliomas are the most common primary malignancy of the central nervous system and are derived from supporting glia. Approximately 60-90% of low-grade gliomas and secondary glioblastomas harbor a heterozygous R132H mutation in the gene coding for isocitrate dehydrogenase 1 (IDH1) [36]. Consistent with other research results, our results showed that the expression of PC increased significantly in the IDH1-mutant U87 cells compared with the wild-type IDH1 U87 cells [10]. Importantly, NDRG2 more significantly inhibited PC expression in the IDH1-mutant U87 cells compared with the wild-type IDH1 U87 cells. Thus, NDRG2 may be a potential therapeutic target for gliomas, especially IDH1-mutant gliomas.

In conclusion, our findings provided the first evidence that NDRG2 interacts with pyruvate carboxylase and induces the degradation of pyruvate carboxylase in glioma cells under glutamine deprivation or glutamine blockade condition. Therefore, NDRG2 inhibits PC-mediated and glucose-dependent anaplerosis in gliomas. Furthermore, IDH1 mutation caused the decrease of α-ketoglutarate and compensatory increase of PC, NDRG2 might block the enhancement of PC and PC-mediated anaplerosis in IDH1-mutant glioma cells. As a result, NDRG2 cooperated with glutamine inhibitor to significantly suppress the growth and proliferation of gliomas. Therefore, NDRG2 combined with glutamine blockade might be a potential treatment strategy for molecular targeted therapy of gliomas.

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Disclosure of conflict of interest

None.

Address correspondence to: Lan Shen, The State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, The Fourth Military Medical University, Changlexi Road 169, Xi'an 710032, Shaanxi, China. Tel: +86-29-8477-4513; Fax: +86-29-84773947; E-mail: lanshen@fmmu.edu.cn

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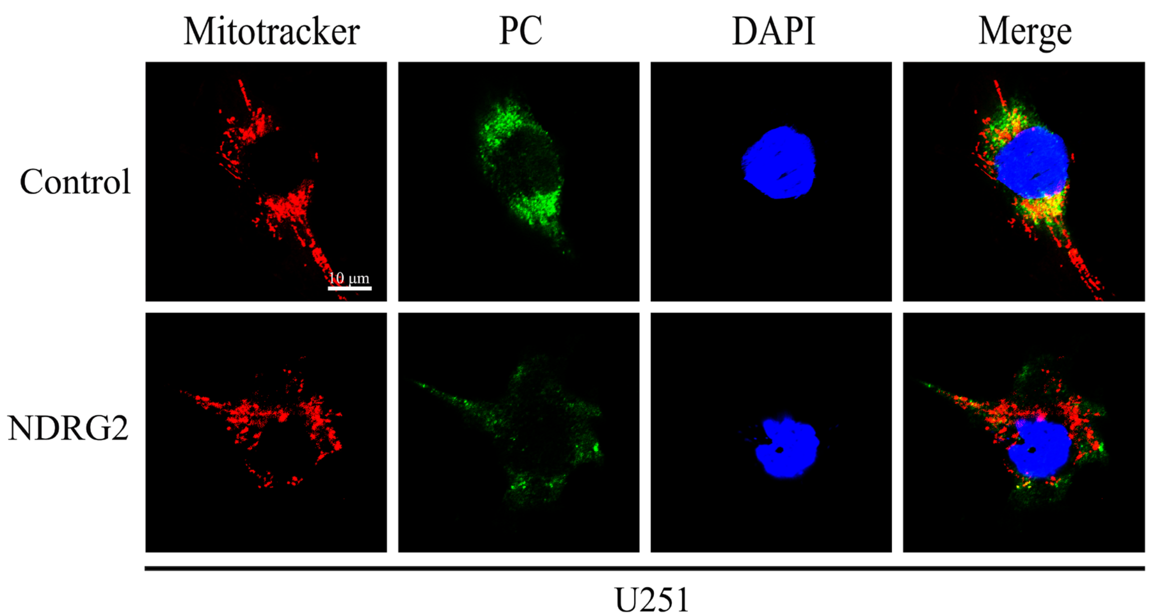
Supplementary Table 1. Primers used for real time-PCR

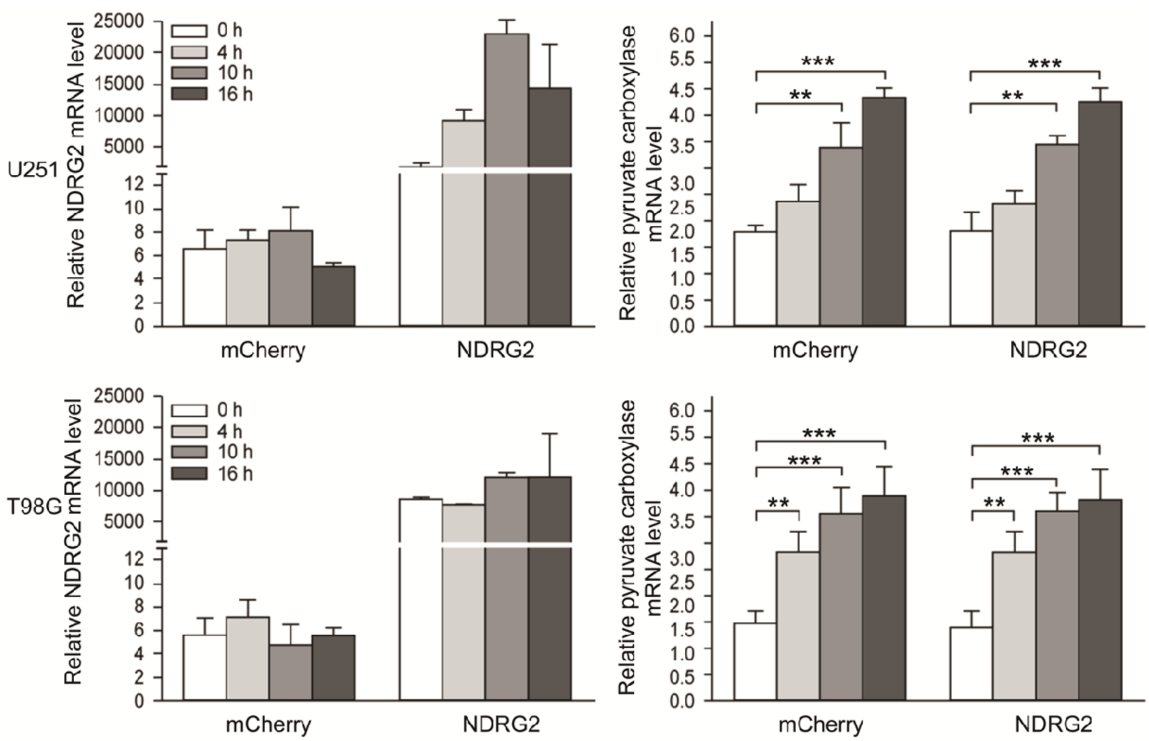
	Forward	Reverse
PC	GCTGGAGGAGAATAACACCCG	GGATGTTCCCATACTGGTCCC
NDRG2	CAGGACAAACACCCGAGACT	AGCCATAAGGTGTCTCCACAG
β-actin	TGTACGTTGCTATCCAGGCTGTG	TCGGTGAGGATCTTCATGAGGTA

Supplementary Table 2. Primers used for PCR

	Forward	Reverse
NDRG2	CCGGCGGCCGCATGGCGGAGCTGCAGGAGG	CCGTCTAGATCAACAGGAGACCTCCATGGT



Supplementary Figure 1. Subcellular localization of pyruvate carboxylase in U251 cells. Immunocytochemical staining of U251 cells was performed using a PC antibody, and visualized using a FITC-conjugated secondary antibody to show the localization of PC (green). MitoTracker®Red CMXRos staining (red) reveals the cell mitochondria, and DAPI staining (blue) reveals the cell nuclei. Scale bar, 10 μm.



Supplementary Figure 2. The mRNA level of pyruvate carboxylase in NDRG2 overexpressing glioma cells. U251 and T98G cells were infected with NDRG2 or mCherry lentivirus for 48 hours, and then cultured in DMEM medium without glutamine for the indicated periods. The mRNA levels of pyruvate carboxylase and NDRG2 at different times were detected by quantitative real-time PCR. All data shown are the mean ± SD of three independent experiments. **P*<0.05, ***P*<0.01.