

Pyruvate kinase M2 is a target of the tumor-suppressive microRNA-326 and regulates the survival of glioma cells

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Emerging studies have identified microRNAs (miRNAs) as possible therapeutic tools for the treatment of glioma, the most aggressive brain tumor. Their important targets in this tumor are not well understood. We recently found that the Notch pathway is a target of miRNA-326. Ectopic expression of miRNA-326 in glioma and glioma stem cells induced their apoptosis and reduced their metabolic activity. Computational target gene prediction revealed pyruvate kinase type M2 (PKM2) as another target of miRNA-326. PKM2 has recently been shown to play a key role in cancer cell metabolism. To investigate whether it might be a functionally important target of miR-326, we used RNA interference to knockdown PKM2 expression in glioma cells. Transfection of the established glioma and glioma stem cells with PKM2 siRNA reduced their growth, cellular invasion, metabolic activity, ATP and glutathione levels, and activated AMP-activated protein kinase. The cytotoxic effects exhibited by PKM2 knockdown in glioma and glioma stem cells were not observed in transformed human astrocytes. Western blot analysis of human glioblastoma specimens showed high levels of PKM2 protein, but none was observed in normal brain samples. Strikingly, cells with high levels of PKM2 expressed lower levels of miR-326, suggestive of endogenous regulation of PKM2 by miR-326. Our data suggest PKM2 inhibition as a therapy for glioblastoma, with the potential for minimal toxicity to the brain.

Keywords: AMP-activated protein kinase, brain tumor, glioma, microRNA, microRNA-326, pyruvate kinase M2, tumor stem cells.

High-grade gliomas are aggressive brain tumors in adults that are untreatable with conventional available therapies. Recent studies have identified microRNAs (miRNAs) as a possible therapeutic tool against these tumors.^{1–4} miRNAs are endogenously expressed noncoding RNAs that are transcribed as primary miRNAs, which are processed by Drosha in the nucleus to pre-miRNAs and then by Dicer in the cytoplasm to mature miRNA duplexes. One strand of the mature miRNA is coupled into the RNA-induced silencing complex and binds the 3'-untranslated region (3'-UTR) of its target messenger RNA (mRNA).⁵ This imperfect binding leads to gene expression regulation through either the inhibition of translation or, in some cases, mRNA degradation.⁶

miRNAs have been recently implicated in the regulation of tumorigenesis, differentiation, proliferation, and survival through the inhibition of major cellular pathways. For instance, miR-7 regulates the Akt and epidermal growth factor receptor (EGFR) pathways, and miR-34a is a key downstream regulator of p53 effects.^{3,4,7–9} We recently showed that miR-326 has tumor-suppressive properties in glioblastoma cells. When we performed a quantitative PCR analysis, we found that miR-326 was downregulated in glioblastoma samples compared with normal brain.¹⁰ Also, the ectopic expression of miR-326 in established glioma cell lines and glioma stem cell lines decreased the expression of Notch-1 and -2, their metabolic activity, and survival.¹⁰ Re-expression of Notch-1 devoid of a 3'-UTR rescued glioma cells from miR-326 toxicity.¹⁰ The above observations that miR-326 regulates the metabolic activity of glioma and glioma stem cells indicate that metabolic pathways are important targets of miRNAs in these cells. Little is known,

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however, about the regulation of metabolic pathways by miRNAs in tumors.

Cancer cells, in contrast to normal cells, are rapidly growing cells that take up nutrients at higher rates to keep up with their rapid catabolic and anabolic needs. This involves reprogramming of their metabolic pathways.¹¹ For instance, cancer cells re-express the embryonic isoform of pyruvate kinase (PK), PKM2, which dephosphorylates phosphoenolpyruvate (PEP) to pyruvate.^{11–13} The expression of PKM2 serves as a metabolic advantage in that it allows the tumor cells to use phosphometabolites upstream of pyruvate as precursors for the synthesis of amino acids, nucleic acids, and lipids that then serve as building materials for the formation of new cells. Interestingly, they still maintain their normal glycolytic pathway for energy production.¹¹

There are four isoforms of PK in mammals: the L- and R-forms are expressed in the liver and red blood cells; the M1 is expressed in almost all adult tissues; and the M2 splice variant is expressed during embryonic development, in undifferentiated tissues, and in tumors.^{11,12,14,15} Unlike PKM1, PKM2 is regulated by tyrosine phosphorylation and differs from the M1 variant in 23 of 531 amino acids.^{11,16} It exists in either a tetrameric form with high affinity for its substrate PEP or a dimeric form with low affinity for PEP. The ability of PKM2 to utilize glucose carbons yielding pyruvate, lactate, and ATP for energy use depends on the tetrameric form, whereas PKM2 generation of structural substrates depends on the dimeric form.^{17,18} This implies that tumor cells (fast dividing cells) that express PKM2 are continuously switching between the high- and low-affinity states for PEP, since anabolic and catabolic processes (the generation of building blocks for new cells and energy) are carried out almost at the same time. In support of this idea, PKM2 was found to be required for the proliferation and survival of some tumors *in vivo*.¹²

The functions of PKM2 and the effects of its inhibition in brain tumors are not yet known. In this study, we showed that PKM2 is a direct target of the tumor-suppressive miR-326 and that it is expressed in established glioma cell lines, brain tumor stem cells, and transformed human astrocytes. Glioma cells with high levels of PKM2 expressed lower levels of miR-326, suggestive of endogenous regulation of PKM2 by miR-326. It is also expressed in primary glioblastoma samples but absent in normal brain temporal lobe. siRNA knockdown of PKM2 leads to decreased glioma cell and glioma stem cell proliferation, invasiveness, clonogenicity, and survival. Furthermore, PKM2 knockdown impaired glioma cell metabolism, with decreased glutathione and ATP levels and increased activation of AMP-activated protein kinase. Since PKM2 is expressed in glioma cells and is important for their survival, but is lacking in the normal brain, its manipulation may serve as a brain-sparing therapy for glioblastoma.

Materials and Methods

Cell Culture

Glioma cell lines U87MG, U251MG, T98G, U373MG, and A172 were all acquired from American Type Culture Collection (ATCC), and the transformed human astrocyte cell line was a kind gift from Dr Russ Pieper, University of California–San Francisco, California. The tumor stem cell lines 0308 and 1228 (Tsc-0308 and Tsc-1228) were derived and validated as described previously.¹⁹ All cell lines were grown under previously described conditions.²⁰

Materials

Pre-miR-control and pre-miR-326 were purchased from Applied Biosystems/Ambion; PKM2 siRNA was from Santa Cruz Biotechnology; and control siRNAs were from Qiagen and Santa Cruz Biotechnology.

Transfection of Cells

PKM2 siRNA, control siRNA, control pre-miRNA, and pre-miRNA-326 transfections were done as described previously using the oligofectamine reagent (Invitrogen) at 20 nM final concentration.²⁰ For Tsc-0308 and Tsc-1228, the plates were first coated with laminin and poly-lornithine (Sigma-Aldrich) and incubated at 4°C for at least 4 hours and washed with 1× phosphate-buffered saline (PBS). Briefly, cells were plated 90 000 per condition in a 6-well plate overnight. They were then washed 3 times with a medium without serum, and 800 µL of Optimem (Invitrogen) was added to each well. To make a final volume of 1000 µL and a final siRNA or pre-miRNA concentration of 20 nM, 200 µL of Oligofectamine, Optimem, and siRNA or miRNA mix was added to the plates. The cells were incubated at 37°C in an atmosphere of 95% air and 5% CO₂ for 6 hours, and media was changed to normal culture media and cultured for 3–6 days. Transfection of PKM2 plasmid lacking 3'-UTR (1 µg/well in a 6-well plate; a kind gift of Dr. Lewis C. Cantley of Harvard Medical School, Boston, Massachusetts) or empty plasmid vector (1 µg/well in a 6-well plate) into U251 cells was done with Eugene HD according to the manufacturer's instruction (Roche Diagnostics). The Eugene HD to DNA ratio used for plasmid transfection was 1:2. For the luciferase assay, U251 cells were transfected with control pre-miR or pre-miR-326 overnight. They were then transfected using Eugene HD (Roche Diagnostics) in 6-well plates with 1.0 µg of PKM2 3'-UTR-reporter plasmid or PKM2 3'-UTR-reporter plasmid with mutations in the binding sites for miR-326 plus 0.05 µg CMV-β-galactosidase, or with 1.0 µg of control-reporter plasmid (parent p-miR-luciferase vector with no insert) plus 0.05 µg CMV-β-galactosidase. PKM1 plasmid was transfected into U251 using the same protocol and reagents as for PKM2.

Plasmid Construction

For the PKM2 3'-UTR-reporter plasmid, this region was first amplified from human genomic DNA using the high-fidelity polymerase enzyme (New England Biolabs) and placed into the multiple cloning site of p-miR-luciferase vector (miRNA expression vector, Biosystems/Ambion) using *HindIII* and *SpeI* restriction enzymes (New England Biolabs).

Primer sequences were as follows:

PKM2-3'-UTR-F: 5'-GTACCTACTAGTT
GGACCCAGAGCCCCTCCTCCAG-3'
PKM2-3'-UTR-R: 5'-GTACCAAAGCTT
CTGGAGGTGCTGCAGTAGTGGGGG-3'

Site Mutagenesis of PKM2 3'-UTR-Luciferase-Reporter Plasmid

For the mutation of miR-326 binding sites on the PKM2 3'-UTR, 25–100 ng of PKM2 3'-UTR-luciferase-reporter plasmid was used as a template. PCR was done as recommended in the Quik-change kit (Agilent, Stratagene), protocol: initialization, 95°C for 30 seconds; denaturing 95°C for 30 seconds; annealing 55°C for 1 minute; and extension/elongation 68°C for 7.5 minutes, and the length of cycle was 18.

For the design of primers used in the mutation of miR-326 binding sites in PKM2 3'-UTR, the Stratagene primer design program was used. Three bases in the “seed” region of each of the miR-326 binding sites in the PKM2 3'-UTR-luciferase-reporter plasmid were changed from CCC to AAA or GCC to AAA (Fig. 1C). The primers used were as follows:

Site 174–193:

PKM2-3'-UTR MUTANT-F-1: 5'-CTGCTCTAGT
GGACAGAAAAGAGCCTGGCTGCCCAT-3'
PKM2-3'-UTR MUTANT-R-1: 5'-ATGGGCAGCC
AGGCTCTTTTCTGTCCCACTAGAGCAG-3'

Site 324–343:

PKM2-3'-UTR MUTANT-F-2: 5'-CCAGTTCCTT
TAGAAAAATGGATGAAAAGAGGACTCCCAA
CCCTGG-3'
PKM2-3'-UTR MUTANT-R-2: 5'-CCAGGGTTG
GGAGTCCTCTTTTCATCCATTTTTTCTAAAGG
AACTGG-3'

Real-Time PCR

Cells were lysed using Qiazol (Qiagen) and then transferred to QIAshredder columns (Qiagen) and centrifuged at 13 000 g for 3 minutes, and RNA was isolated using the miRNeasy kit according to the manufacturer's instructions (Qiagen). RT-PCR on 500 ng of RNA using the miScript reverse transcription kit (Qiagen) was used to generate cDNA. From 96 ng of cDNA template, quantitative real-time PCR analyses

for miR-326 and U6B were performed using miR-326- and U6B-specific forward primers and a universal reverse primer according to the manufacturer's protocol (Qiagen). U6B was used as a control to normalize the levels of miR-326. Applied Biosystems (StepOnePlus) real-time PCR system was used to carry out the quantitative PCR, using hot start, with annealing at 55°C (30 s), extension 70°C (30 s) for 40 cycles, followed by a melt curve analysis. Data analysis for the differences in miR-326 expression was carried out using StepOne software v2.1 (Applied Biosystems).

Determination of Cell Numbers (Cell Count)

Glioma cells transfected with either control pre-miR or pre-miR-326 were cultured for 3–6 days. They were then washed with 1× PBS and trypsinized, washed again with 1× PBS, and resuspended in 3 mL of 1× PBS, and the number of cells was determined with microscopy (Carl Zeiss, Axiovert 40 C) using a hemocytometer.

Protein Extraction and Quantification

Cells were lysed in 100–200 µL of 1× cell lysis buffer containing 0.2% SDS and complete protease inhibitors (Cell Signaling Technology). Cell debris was removed from the extracts by centrifugation at 10 000 g for 3 minutes and the clear protein lysate transferred to new tubes.

For protein assay/quantification, the BSA standards of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 2 µg/µL were prepared and placed into 7 wells in a 96-well plate and the volume adjusted to 20 µL. Five microliter of samples was also placed into other wells in a 96-well plate with the BSA standard and volume adjusted to 20 µL. Two hundred twenty-five microliter of bicinchoninic acid (Bio-Rad) mix was added to each well and incubated for 5 minutes in the dark at room temperature. The color change was measured at 595 nm using a spectrophotometer (Biotek Elx808). The absorbance of the standards versus concentration was plotted, the extinction coefficient was computed, and the protein concentrations of the unknown samples were calculated using KC Junior software (Biotek).

Immunoblots

Patient samples were placed in a tissue bank with approval from the University of Virginia Institutional Review Board and were used here in a deidentified fashion. Immunoblots were performed as described previously.²⁰ Primary antibodies included anti-PKM2 antibody (a kind gift from Dr. Lewis C. Cantley of Harvard Medical School, Boston, MA), anti-PKM1 (a kind gift from Dr. Mathew Vander Heiden of Koch Institute at MIT, Cambridge, MA), anti-poly-ADP ribose polymerase (PARP), anti-α-tubulin (11H10), and rabbit polyclonal immunoglobulin G (IgG) antibody directed against phospho-(Thr172)-AMPK (AMP-activated protein kinase) and phospho-(Tyr105)-PKM2 (Cell Signaling

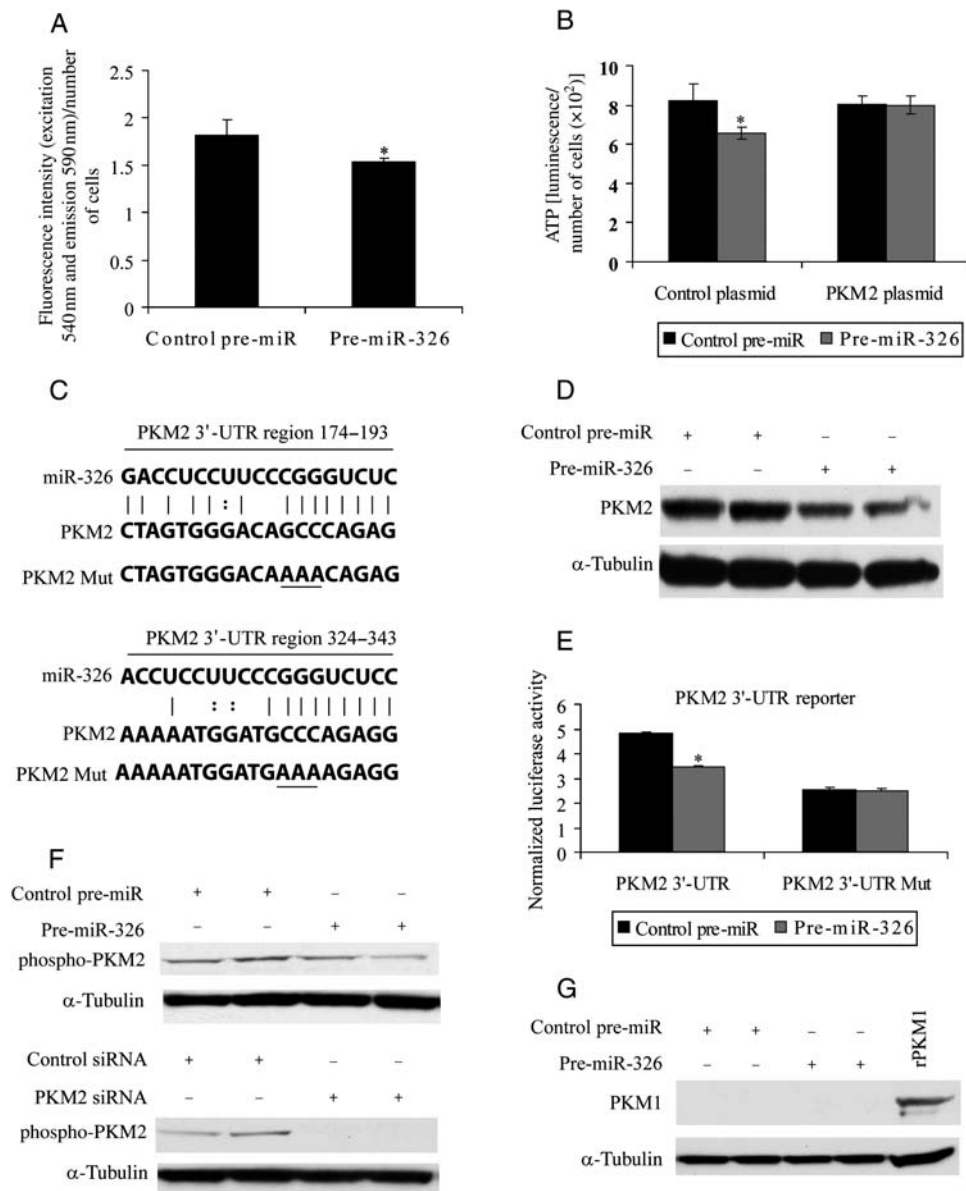


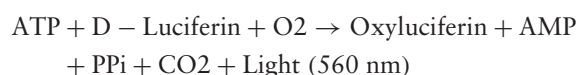
Fig. 1. miR-326 targets PKM2 and decreases the metabolic activity with decreased ATP levels in glioma cell lines. (A) Plots of AlamarBlue assay for the metabolic activity of the U251MG cell line 3 days after transfection with control pre-miR or pre-miR-326 (* $P < .05$). The AlamarBlue assay was normalized (divided by) with cell numbers. (B) A plot of ATP levels in the U251 glioma cell line normalized (divided by) with cell numbers 3 days after transfection with control pre-miR or pre-miR-326 alone and 3 days together with an empty plasmid vector or an PKM2 plasmid lacking 3'-UTR (* $P < .05$). (C) Sites of miR-326 seed matches in the PKM2 3'-UTR, with or without mutations in 3 bases (CCC changed to AAA or GCC changed to AAA) in each of the sites. (D) Immunoblots showing the protein expression of PKM2 in U251MG cells 3 days post-transfection with control pre-miR or pre-miR-326 (α -tubulin loading controls shown at the bottom). (E) Normalized activity of PKM2 3'-UTR-luciferase-reporter plasmid or mutant PKM2 3'-UTR-luciferase-reporter plasmid, 3 days post-transfection with control pre-miR or pre-miR-326 (* $P < .05$). (F) Immunoblots showing the phosphorylation of PKM2 at Tyr¹⁰⁵ in U251MG cells 3 days post-transfection with control pre-miR or pre-miR-326 or control siRNA or PKM2 siRNA (α -tubulin loading controls shown at the bottom). (G) Immunoblots showing the expression of PKM1 protein levels in U251MG cells 3 days post-transfection with control pre-miR or pre-miR-326 or rat PKM1 plasmid (α -tubulin loading controls shown at the bottom).

Technology). Horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse IgG were used (1:10 000; Jackson Immunology Labs). Protein band quantification was performed using the NIH-ImageJ program.

ATP Assay

U251MG cells transfected with either control siRNA or PKM2 siRNA or control pre-miR or pre-miR-326 or A172, Tsc-1228, and Tsc-0308 cells transfected with

either control siRNA or PKM2 siRNA were washed with 1 × PBS (Fisher Scientific) once, trypsinized and washed again once in 1 × PBS, and resuspended in 100 µL of 1 × PBS. Forty microliter of the cells was mixed with 40 µL of CellTiter-Glo luminescent reagent (Promega) and incubated for 15 minutes with agitation at room temperature. The luciferase activity was measured using a Luminometer (Promega) as described previously.²⁰ Ten microliter was taken from the remaining unused 60 µL of 1 × PBS containing cells, and cell numbers were determined by microscopy using a hemocytometer. The measured ATP was normalized by dividing with cell numbers. This assay uses recombinant luciferase to catalyze the reaction below:



Because ATP is the limiting component in the luciferase reaction, the intensity of the emitted light is proportional to the ATP concentration. The measurement of the light intensity using a luminometer permits direct quantification of changes in cellular ATP levels.²¹

AlamarBlue Assay

Cells transfected with either control pre-miR or pre-miR-326, or control siRNA or PKM2 siRNA, were incubated for 3–6 days. AlamarBlue (Invitrogen) was added in a ratio of 1:10 and incubated for 1–2 hours. One hundred microliter of the AlamarBlue media mixture was placed in a 96-well plate, and the fluorescence determined with a fluorescence plate reader at excitation of 540 nm and emission of 590 nm. This assay is based on the conversion of a blue nonfluorescent dye resazurin to a pink fluorescent dye resorufin by cells. Cells with decreased metabolic activities lack the ability to perform the above-mentioned conversion, yielding a reduction in fluorescence readout.

Glutathione Assay

U251MG, A172, Tsc-1228, and Tsc-0308 cells transfected with either control siRNA or PKM2 siRNA were washed with 1 × PBS once, trypsinized and washed again once with 1 × PBS, and resuspended in 100 µL of PBS. Forty microliter of the cells were mixed with 40 µL of glutathione reagent (GSH-Glo; Promega) and incubated for 30 minutes at room temperature with agitation. One hundred microliter of luciferin reagent was added and further incubated at room temperature for 15 minutes with continuous agitation, and luciferase activity was measured using a luminometer (Promega) as described previously.²⁰ Ten microliter was taken from the remaining unused 60 µL of 1 × PBS containing cells, and cell numbers were determined by microscopy using a hemocytometer. The measured glutathione was normalized by dividing with cell numbers. This assay is based on the conversion of a luciferin derivative into luciferin in the presence of

glutathione present in cell lysate (sample), catalyzed by glutathione-S-transferase present in the assay reagent. The signal generated in a coupled reaction with firefly luciferase captured and read by the luminometer is proportional to the amount of glutathione present in the sample (cell extract).

Caspase-3/7 Assay

Cells transfected with either control siRNA or PKM2 siRNA were washed with 1 × PBS once, trypsinized and washed again once with 1 × PBS, and resuspended in 100 µL of PBS. Forty microliter of the cells were mixed with 40 µL of caspase-Glo-3/7 reagent (Promega) and incubated for 1 hour at room temperature with agitation. The luciferase activity was measured as described previously using a luminometer and normalized with protein concentration.¹⁰

Luciferase Assay

Luciferase reporter assays were performed as described previously on a Promega Glomax 20/20 luminometer.²⁰ PKM2 3'-UTR-luciferase or PKM2 3'-UTR-luciferase mutant (with mutation in 3 bases each on each of the two miR-326 binding sites) activities were double normalized by first dividing the luciferase activity in each well by β-galactosidase activity, then dividing the luciferase/β-galactosidase activity by that from a parallel set done with parent luciferase vector (p-miR-luciferase vector) with no insert in the multiple cloning site.

Cell Invasion Assay

Invasion was determined by the modified Boyden chamber assay with a 8-µm pore size polycarbonate filter (Becton Dickinson) coated with type IV collagen (Sigma). Prepared cell suspension transfected with control siRNA or siRNA directed at PKM2 (300 µL; 0.3×10^6 – 0.5×10^6 cells/mL) in RPMI medium containing 0.1% fetal bovine serum was added to the upper compartment of each insert. After 8 hours of incubation, the filters were fixed and stained with 0.1% crystal violet solution. The invaded cells that passed through the filter to the lower surface of the membrane were counted under the microscope by counting at least three high-power fields. Each sample was assayed in triplicate, and assays were repeated at least twice. Quantification of the invasion assay was done as described previously.³

Soft Agar Colony Forming Assay

A 0.6% agar medium base layer was made, and 3 mL was added to each well (6-well plate) to prevent the cells from attaching and forming a monolayer on the plastic substrate. One milliliter of culture medium containing cells transfected with either control siRNA or PKM2 siRNA was mixed with 1 mL of 0.6% agar medium, poured on the base layer and allowed to

solidify, and immediately placed in an incubator at 37°C and 5% CO₂. Medium was changed every 2–3 days, and after 21 days, cells were stained with Wright's stain and counted under the microscope (Carl Zeiss, Axiovert 40 C).

Statistical Analysis of Data

Data presented in figures as bar graphs are always expressed as standard \pm SEM (standard error of mean). For calculating the statistical significant differences between the groups of data, the Student's two-way pair or unpaired *t*-test was used, and the linear regression analysis (sigma plot) was used for Fig. 2C ($P \leq .05$, linear correlation [r] = 0.965).

Results

miR-326 Targets PKM2 and Decreases ATP Levels in Glioma

We previously showed that miR-326 targets the Notch pathway, is toxic to glioma cells, and affects their metabolic activity with decreased ATP levels¹⁰ (Fig. 1A and B). When we searched for its possible metabolic targets, we found two sites with 1–8 seed matches in the 3'-UTR of PKM2 (Fig. 1C), recently implicated in tumorigenesis.¹² In addition, transfection of PKM2 plasmid lacking 3'-UTR into the U251 glioma cell line rescued the decrease in ATP levels caused by miR-326 (Fig. 1B). To investigate whether PKM2 is a functional target of miR-326, we assessed its protein levels. Immunoblot indicated decreased levels of PKM2 protein in pre-miR-326 transfected cells when compared with cells transfected with control pre-miR (Fig. 1D). Glioma cells transfected with pre-miR-326 exhibited a significant decrease in the luciferase activity ($P < .05$) of a reporter plasmid bearing the 3'-UTR of PKM2 when compared with control pre-miR transfected cells (Fig. 1E). Mutations in 3 bases each in the "seed" complementary sites for miR-326 completely rescued the repression of PKM2 3'-UTR-luciferase activity (Fig. 1C and E). These data indicated that PKM2 is a direct and functional target of miR-326. It has been recently reported that the phosphorylation of PKM2 on tyrosine residue 105 (Y¹⁰⁵) by growth factors is critical for its oncogenic and tumorigenic effects.^{16,22} Since miR-326 decreased the total PKM2 protein levels and decreased the growth of glioma cells,¹⁰ we assessed the effects of this miRNA on the phosphorylation state of PKM2 in U251 cells. Transfection of U251 cells with pre-miR-326 decreased the phosphorylation of PKM2 at Tyr¹⁰⁵ (Fig. 1F). PKM2 siRNA was used as a positive control for the detection of phosphor-Tyr¹⁰⁵ (Fig. 1E). Immunoblot analysis showed no detectable expression of PKM1 in the U251 glioma cell line (Fig. 1G).

PKM2 Expression Is Restricted to Glioblastoma and Proliferating Cells and Negatively Correlates with MiR-326 Levels

The expression of PKM2 has been suggested to be restricted to tumors and rapidly dividing cells.^{11,12,14} To confirm this finding in glioma, we obtained tissue samples from brain tumor (glioblastoma) patients (8 samples) and compared its expression in these tissues to normal brain temporal lobe (9 samples from epilepsy surgeries; Fig. 2A). We also compared the expression of PKM2 in transformed human astrocytes, established glioma cell lines, and glioma stem cells using immunoblot (Fig. 2B). PKM2 was highly expressed in glioblastoma tissues, transformed human astrocytes, established glioma, and glioma stem cells but absent in normal brain temporal lobe (Fig. 2A and B). We found differences in the protein levels of PKM2 in the cell lines compared and also, since the transfection of glioma cells with miR-326 reduced PKM2 protein levels (Fig. 1D), it suggested the possibility of a negative correlation between the expression of miR-326 and PKM2. We selected cells with high and low levels of PKM2 and evaluated the correlations of miR-326 and PKM2 (Fig. 2C). Cells with high levels of PKM2 had lower levels of miR-326 and vice versa (Fig. 2C). These data suggest miR-326 as a possible endogenous regulator of PKM2 expression.

Effects of PKM2 siRNA on Glioma Cell Metabolic Activity, Proliferation, and Survival

Given that PKM2 is selectively expressed in glioblastomas and not in the normal brain (Fig. 2A) and that it has been implicated in the survival of other tumors,¹² we assessed its importance for the viability of glioma cells. This was tested both in the standard glioblastoma cell lines and in the glioblastoma stem cell lines, representing a critical tumorigenic and treatment-resistant subpopulation of cells within gliomas. We first demonstrated by immunoblotting the efficiency of a PKM2 siRNA in the established glioma cell lines, transformed astrocytic cells, and glioma stem cells (Fig. 3A). Given its effective knockdown of PKM2 protein expression, we assessed the effects of this siRNA on the metabolic activity, proliferation, and survival of these cells. First, metabolic activities of cells were determined using AlamarBlue, 3 days post-transfection, a time point with no PKM2 siRNA toxicity. The AlamarBlue assay, a redox assay that determines cellular metabolic activity, was significantly decreased ($P < .05$) in PKM2 siRNA transfected cells (Fig. 3B). Also, cell numbers were decreased in all cells transfected with PKM2 siRNA cultured for 5–6 days when compared with control siRNA (Fig. 3C), indicating that PKM2 siRNA ultimately decreases the glioma cell proliferation or possibly induces their death. However, the decrease in cell number exhibited by PKM2 knockdown was not observed in transformed astrocytes (Fig. 3C). To ascertain the effect of PKM2 siRNA on cell survival, we performed immunoblot for PARP cleavage and measured

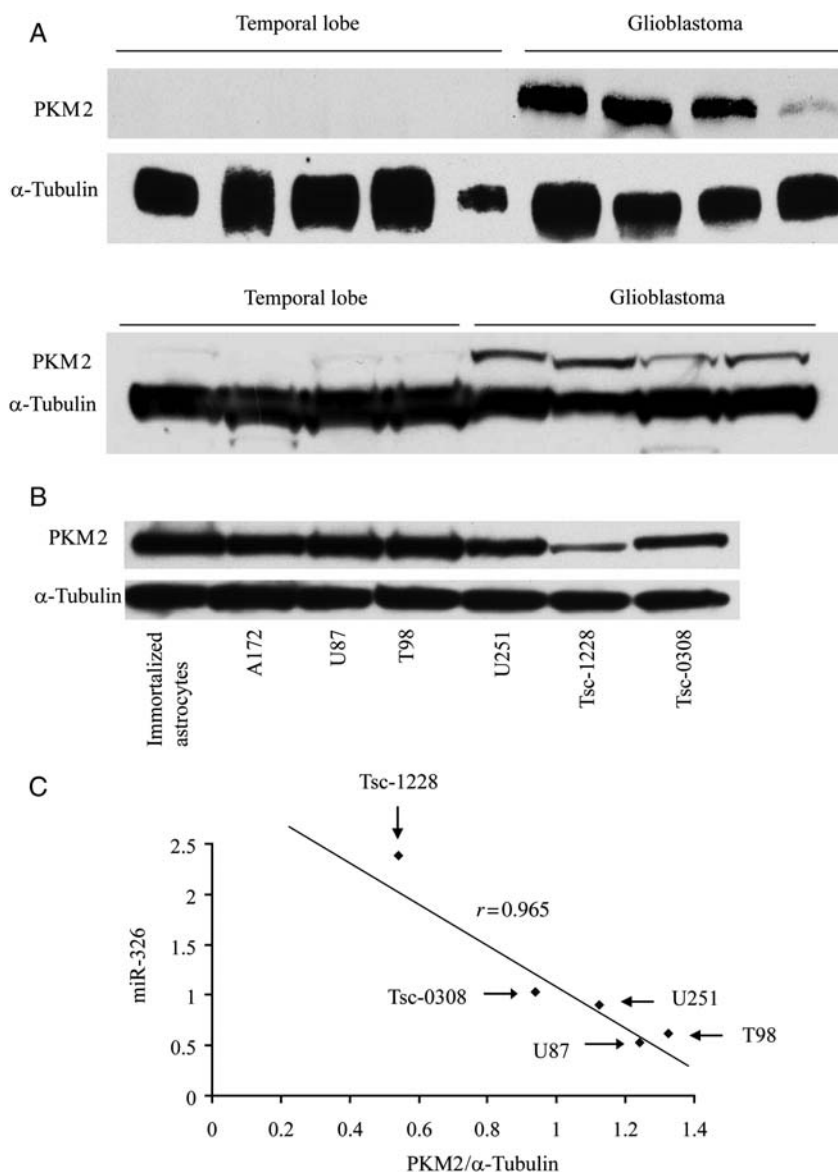


Fig. 2. PKM2 is expressed in glioblastoma samples, transformed human astrocytes, glioma and glioma stem cells and absent in the normal brain temporal lobe and negatively correlates with miR-326 levels. Immunoblot showing the expression of PKM2 protein in (A), 8 glioblastoma patient samples and 9 normal brain temporal lobe (samples from epilepsy surgeries; α -tubulin loading controls shown at the bottom). (B) Lysates from cell lines: transformed human astrocytes, A172, U87MG, T98G, U251MG, and brain Tsc-0308 and Tsc-1228 (α -tubulin loading controls shown at the bottom). (C) A plot of the linear inverse correlation between the expression of PKM2 protein and miR-326 expression in U87, T98, U251, Tsc-1228 and Tsc-0308 cells ($P \leq .05$, linear correlation [r] = 0.965). Immunoblot of PKM2 was quantified using NIH-ImageJ and normalized with quantified (NIH-ImageJ) α -tubulin loading controls. miR-326 expression was quantified by quantitative PCR and normalized with U6B small RNA.

caspase-3/7 activation, 2 well-known hallmarks of apoptosis.²³ PKM2 siRNA increased caspase-3/7 activity and induced PARP cleavage into its characteristic 89 kDa fragment in glioma cells (Fig. 3D and E).

Effects of PKM2 Knockdown on Glutathione Levels, ATP Levels, and AMP-Kinase Activation

Cellular proliferation and apoptotic cell death are regulated by a sophisticated interplay between metabolism, the scavenging potential of proteins such as glutathione,

and cellular signaling.^{24,25} PKM2 is an important metabolic protein that ensures that transformed cells have readily available either the ATP required for energy or the building blocks needed at that given moment.¹⁷ Given this, as well as our previous results, we assessed whether PKM2 knockdown would affect ATP and glutathione levels in glioma. Glioma cells transfected with PKM2 siRNA exhibited lower amounts of ATP and glutathione levels (Fig. 4A and B). Since a decrease in the ATP levels in cells normally leads to increased AMP:ATP ratios, we hypothesized that AMPK would be activated

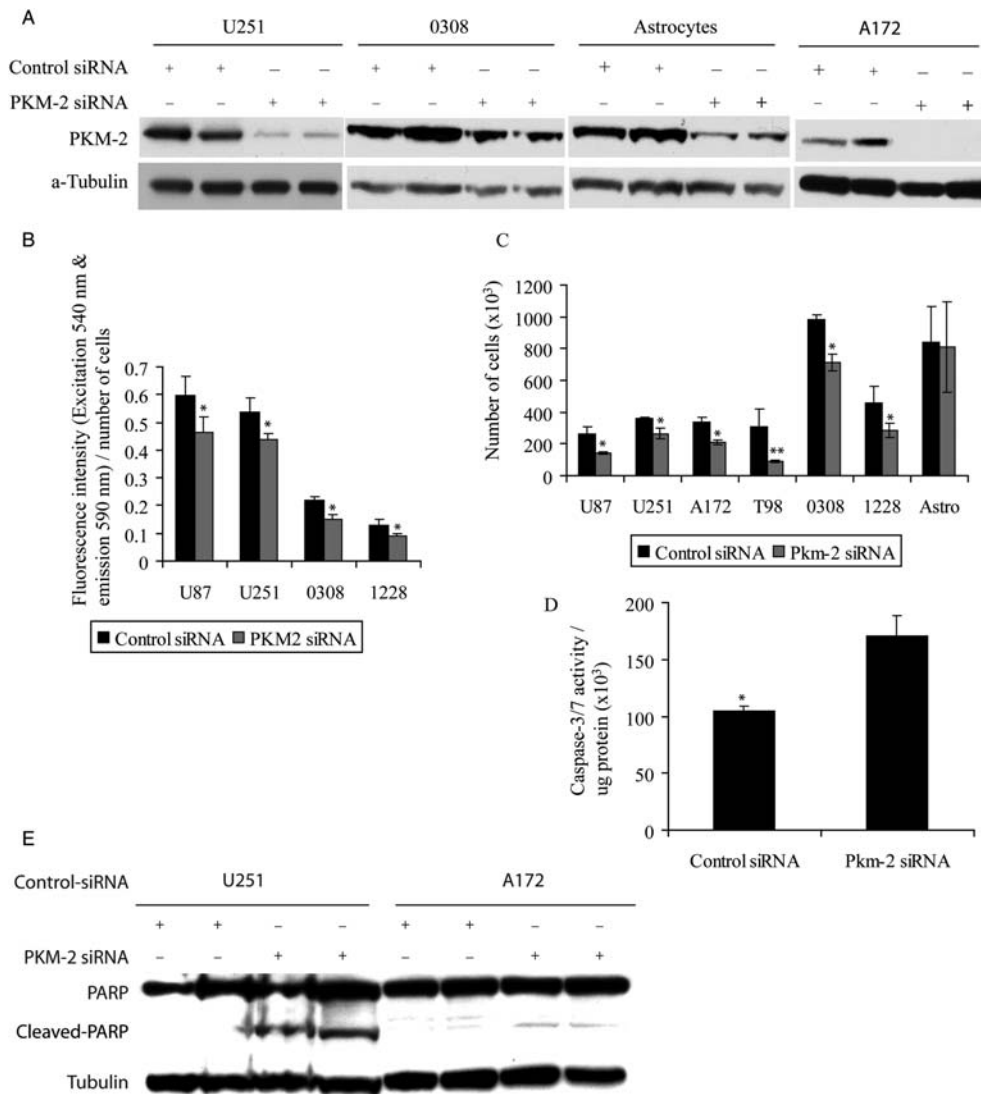


Fig. 3. Effect of PKM2 siRNA on its protein expression and the survival and metabolic activity of glioma and glioma stem cells. (A) Immunoblots showing the protein expression of PKM2 in U251MG, transformed human astrocytes, A172 and 0308 glioma stem cells 3 days after transfection with control siRNA or PKM2 siRNA (α -tubulin loading controls shown at the bottom). (B) Plots of AlamarBlue assay for the metabolic activity normalized (divided) with cell numbers of U87MG and U251MG cell lines and glioma Tsc-0308 and Tsc-1228 3 days post-transfection with control siRNA or PKM2 siRNA (* $P < .05$). (C) A plot of cell counts performed 5–6 days after transfection of U87MG, U251, A172, and T98G cell lines, transformed human astrocytes, and Tsc-0308 and Tsc-1228 with control siRNA or PKM2 siRNA (* $P < .05$, ** $P < .01$). The established glioma cell lines transfected with PKM2 siRNA undergo apoptosis demonstrated by caspase activity and cleaved PARP. (D) A plot of caspase-3/7 activity normalized with protein concentration in U251MG glioma cell lines 4 days after transfection with control siRNA or PKM2 siRNA (* $P < .05$). (E) Immunoblots of full-length and cleaved PARP protein in U251MG and A172 glioma cell lines 6 days after transfection with control siRNA or PKM2 siRNA. Bottom: α -tubulin as a loading control.

with PKM2 gene knockdown (Fig. 3A). Indeed, glioma cells transfected with PKM2 siRNA exhibited a significant increase in AMPK phosphorylation (Fig. 4C). AMPK phosphorylation is known to have anticancer effects such as inhibiting the oncogenic mTOR protein.^{26,27}

Effects of PKM2 Knockdown on Glioma Cell Invasion and Colony Formation

Besides proliferation, invasiveness is another hallmark of aggressive glioma. We tested the effect of PKM2

siRNA transfection on the invasiveness of glioma cells in vitro using U251 cells. Transfection of an established glioma cell line with PKM2 siRNA decreased invasion through collagen IV-coated transwells (Fig. 5A). We further examined if PKM2 gene knockdown would affect the clonogenic growth of glioma cells. Glioma cells transfected with PKM2 siRNA developed significantly ($P < .05$) fewer and smaller colonies, indicating that PKM2 gene knockdown decreases both invasiveness and clonogenicity of glioma cells (Fig. 5B).

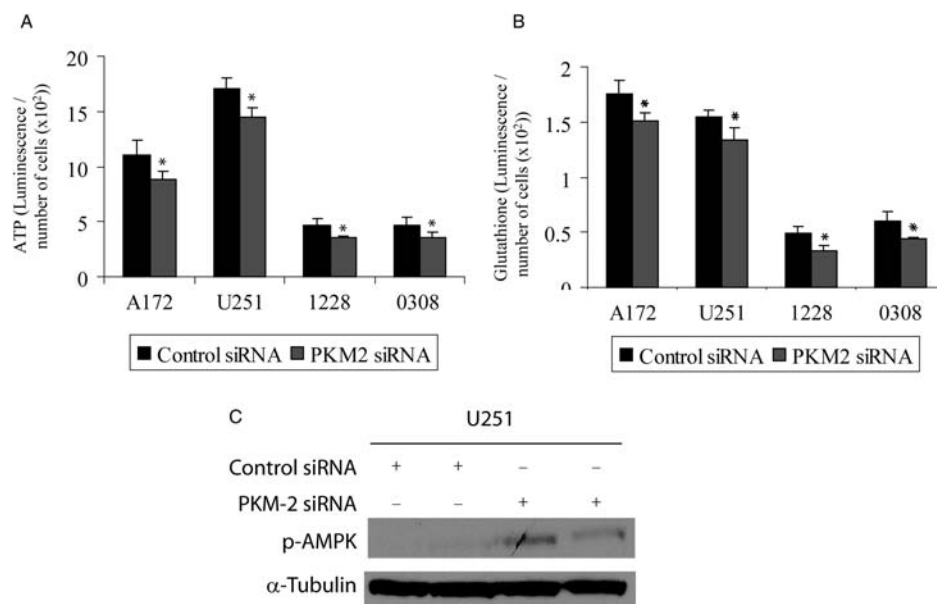


Fig. 4. PKM2 siRNA knockdown reduced ATP and glutathione levels of glioma cells and activated AMPK. (A) A plot of ATP levels in A172 and U251 glioma cell lines and glioma stem cell lines 1228 and 0308 normalized (divided) with cell numbers 3 days post-transfection with control siRNA or PKM2 siRNA ($*P < .05$). (B) A plot of glutathione levels in U251 and A172 glioma cell lines and glioma Tsc-1228 and Tsc-0308 normalized (divided) with cell numbers 3 days after transfection with control siRNA or PKM2 siRNA ($*P < .05$). (C) Immunoblots of phosphorylated AMPK at threonine-172 (phospho-(Thr172)-AMPK; α -tubulin loading controls shown at the bottom).

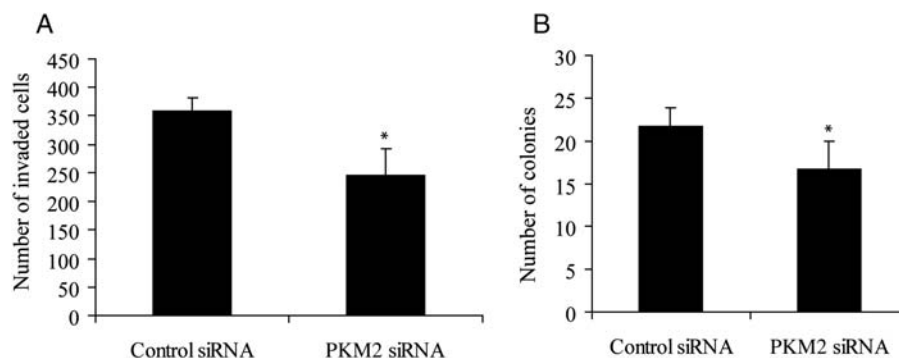


Fig. 5. PKM2 knockdown decreases invasiveness and clonogenicity of U251MG glioma cell line. (A) A plot showing the mean number of invaded U251MG glioma cell line 3 days after transfection with control or PKM2 siRNA using the modified Boyden Chamber assay coated with type IV collagen ($*P < .05$). (B) Plot showing the mean number of colonies formed in soft agar by U251MG glioma cells 3 days after transfection with control siRNA or PKM2 siRNA ($*P < .05$).

Discussion

There are very few studies focused on the regulation of metabolic genes by miRNAs, although these genes are dysregulated and are key elements in the survival of tumors.^{28,29} In our previous work³⁰ and in this study, we show that miR-326 decreased the metabolic activity of glioma with decreased ATP levels. In addition, PKM2 is a direct and functional target of miR-326, suggesting that miR-326 could regulate the glioma metabolism through its downregulation. Transfection of glioma cells with PKM2 plasmid lacking 3'-UTR rescued the decrease in the ATP levels exhibited by miR-326, demonstrating that

PKM2 inhibition mediated the metabolic effects of miR-326. Importantly, we found that PKM2 is expressed in established glioma cell lines, transformed human astrocytes and glioblastoma tissues but absent in the temporal lobe of normal brain. In addition, PKM2 protein levels in glioma cells negatively correlated with miR-326 levels, further confirming that PKM2 is an endogenous target of miR-326. PKM2 gene knockdown by siRNA decreased cell proliferation, survival, invasion, and clonogenicity of glioma cells but did not affect the survival of transformed human astrocytes. Moreover, PKM2 knockdown decreased ATP and glutathione levels and activated AMPK. These results show PKM2 to be a key target

of the tumor-suppressive miR-326 and a potential therapeutic target in its own right in gliomas.

The decrease in glioma proliferation and survival with PKM2 knockdown is in accordance with a recent study in other tumors and tumor cell lines.¹² We also observed that PKM2 is not required for the survival and proliferation of transformed human astrocytes, suggesting that PKM2 may be selectively required for cancer cell metabolism; it is, however, not clear if it is a transforming factor or not.^{12,16} It also implies that transformed cells differ in the genes that are responsible for driving their metabolism and survival. However, further careful studies are necessary to determine what is required along with PKM2 expression for the survival and proliferation of transformed cells and tumors and if PKM2 is a transforming or contributing factor.

Tumors and rapidly dividing cells have several metabolic adaptations that allow them to proliferate and thrive under a variety of nutrient conditions. Such adaptations seem to include the expression of PKM2. PKM2 is responsible for the transfer of phosphate from PEP to ADP, accompanied by the formation of ATP and pyruvate and thus stabilizing energy charge in cells ($[(ATP) + 1/2[ADP]] : ([AMP] + [ADP] + [ATP])$).^{31,32} This means that the depletion of PKM2 will lead to the disruption of the above reaction, with a decrease in ATP levels. We found in this study that PKM2 knockdown decreased the ATP levels in glioma cells, fitting the known function of PKM2.^{33,34} This suggests that glioma cells devoid of PKM2 might have increased AMP:ATP ratios and thus activation of AMPK,³⁵ a kinase detrimental to glioma and other cell types in an activated state.^{26,36–39} We indeed saw an increase in AMPK activation, as well as decreased glutathione levels. This suggests that glioma cells deficient in PKM2 exhibit decreased metabolism and intolerance for oxidative stress, consistent with our observations of glioma cell apoptosis with PKM2 knockdown.

We observed that PKM2 knockdown decreased glioblastoma cell clonogenicity and invasiveness, key

hallmarks of their aggressiveness and incurable nature in patients. Additionally, PKM2 inhibition shows effectiveness not only against standard glioma lines but also against glioblastoma tumor stem cells resistant to the standard therapies. These results suggest PKM2 may have a particular value as a target in glioma therapy.

Our finding that PKM2 is expressed in glioma cell lines, transformed astrocytes, glioblastoma tissues, and brain tumor stem cells, but not in normal brain tissue, is in accordance with a recent report that PKM2 is only expressed in tumors and transformed cells but not in normal cells.^{11,14} These findings further reinforce the therapeutic potential of PKM2 inhibition for glioma.

In conclusion, since PKM2 inhibition is toxic to glioma and glioma stem cells but nontoxic to transformed human astrocyte, and since PKM2 is absent in normal brain and by report in other normal tissues, inhibition of this kinase may have utility against these tumors with minimal side effects in patients.

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