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Pyruvate kinase M2 contributes to cell growth in gastric cancer via aerobic glycolysis

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ABSTRACT

Pyruvate kinase M2 (PKM2) serves as a key enzyme that promotes aerobic glycolysis. This study investigated the function of PKM2 in tumor growth and maintenance in gastric cancer (GC). Histological staining was applied to detect PKM2 expression in GC tissues. PCR and western blotting were used to measure PKM2 expression in GC cells. PKM2 was knocked down to examine the biological behavior of tumors, glycometabolism, and apoptosis. PKM2 was upregulated in GC tissues (65%, 34/52) compared with that in adjacent normal tissues (27%, 10/37). Moreover, *PKM2* knockdown inhibited proliferation of BGC823 GC cells, and elevated PKM2 levels were associated with poor survival of GC patients. Furthermore, knockdown of PKM2 altered the biological behavior of BGC823 cells through induction of apoptosis. In conclusion, the results of this study indicated that inhibition of PKM2 could represent a novel strategy for gastric cancer treatment.

1. Introduction

Gastric cancer (GC) is the leading cause of cancer-related mortality in the world and is one of the most commonly diagnosed cancers [1,2]. As multistep changes are involved in carcinogenesis, numerous targets for GC therapy have been identified. In the 1920s, Otto Warburg observed, for the first time, metabolic alterations in tumor tissues in comparison with normal tissues, known as the "Warburg effect" [3,4]. Since then, many attempts have been made to discover the interactions between metabolism and cancer biology [5–7]. Increasing evidence suggests that cancer could be defined as a metabolic disease [8]. Recently, cancer research has progressed in a novel direction, evaluating enzymes and the role they play in cancer metabolism [9].

Pyruvate kinase (PK) is a key glycolytic enzyme, catalyzing transformation of phosphoenolpyruvic acid (PEP) and ADP to pyruvate and ATP, respectively. In mammals, four isoenzymes of PK have been reported: PKL (liver-type PK), PKR (red blood cell PK), and PKM1 and PKM2 (PK muscle isozyme M1 and M2, respectively) [10]. PKM1 shows high enzymatic activity and is mainly expressed in the muscle and brain, while PKM2 exists in a low-activity form, which is dominant in both normal proliferating cells and cancer cells. Recently, several studies reported a correlation between PKM2 and cancer development [11,12]. Not only does PKM2 serve as a crucial regulator in cancer metabolism [13], but it also exerts a significant influence on the proliferation and apoptosis of cancer cells. Moreover, *PKM2* promotes the process of cancer migration and metastasis [14].

However, the exact role of PKM2 in GC has not yet been fully elucidated. In this research, we explored whether PKM2 level is associated with cancer progression and whether PKM2 suppression inhibits tumor growth and maintenance in GC. Further, the molecular mechanisms underlying the effect of PKM2 on GC were evaluated.

2. Materials and methods

2.1. Tissue array and IHC staining

A total of 52 paraffin-embedded GC tissues were obtained from Beijing Cancer Hospital. This study was approved by the Ethics Committee of Peking University Cancer Hospital and was conducted according to the Declaration of Helsinki. The tissue array was stained with anti-PKM2 antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA), and histological staining was performed using DAB (DAKO, Carpinteria, CA, USA).

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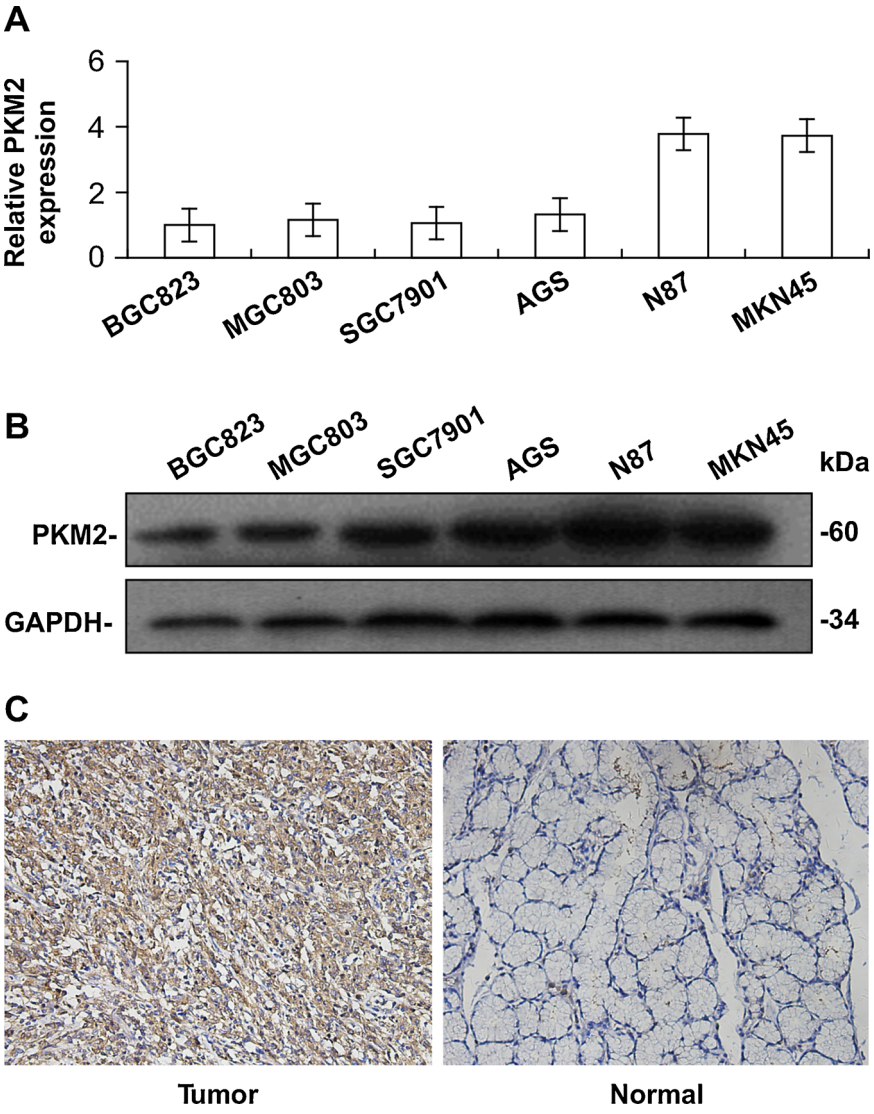

Fig. 1. Differential expression of PKM2 in gastric cancer cells and tissues. (A) *PKM2* mRNA expression was analyzed in 6 gastric cancer cell lines by real-time PCR. (B) PKM2 protein was detected in 6 gastric cancer cell lines by western blotting. (C) Representative images of PKM2 immunohistochemical staining in tissue microarrays (original magnification, 200×). T, gastric cancer tissues; N, adjacent normal tissues.

Table 1
PKM2 expression in gastric cancer.

		PKM2 expression		
Histology	Cases	High, n (%)	Low, n (%)	*P*
Normal	37	10 (27)	27 (73)	< **0.001**
Tumor	52	34 (65)	18 (35)	

2.2. Kaplan Meier-Plotter

Survival information for GC patients with differential expression of PKM2 was analyzed with the KM-Plotter database [15]. Samples from GC patients were separated into two groups based upon median expression of PKM2. A survival plot was generated using two cohorts and log-rank; then, hazard ratio was calculated with a 95% confidence interval.

2.3. Cell culture

Human GC cell lines BGC823, MGC803, and SGC7901 were obtained from China, and AGS, N87, and MKN45 were bought from the American ATCC (Manassas, VA, USA). Cells were cultivated in DMEM (Gibco BRL, Grand Island, NY, USA) and supplemented with 5% fetal bovine serum (FBS, Gibco BRL) in 5% CO_2 at 37 °C.

2.4. Plasmid construction and stable transfection

Primer sequences of PKM2-specific small hairpin RNA (shRNA) were as follows: sense 5′-GATCCGCCTGTCATCTGTGCTACTTTCAAGAGAAGTAGCACAGATGACAGGCTTTTTTGGAAA-3′; antisense 5′-AGCTTTTCCAAAAAAGCCTGTCATCTGTGCTACTTCTCTTGAAAGTAGCACAGATGACAGGCG-3′.

PKM2 shRNA was cloned into the pSliencer 3.0-H1 (+) vector (BamH1/HindIII sites; Invitrogen, Carlsbad, CA, USA). BGC823 cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen), and G418 was used for stable selection of *PKM2* shRNA-expressing cells.

2.5. Cell proliferation test

Cell proliferation was detected by MTT assay. *PKM2* shRNA-expressing cells and mock cells were seeded in 96-well plates,

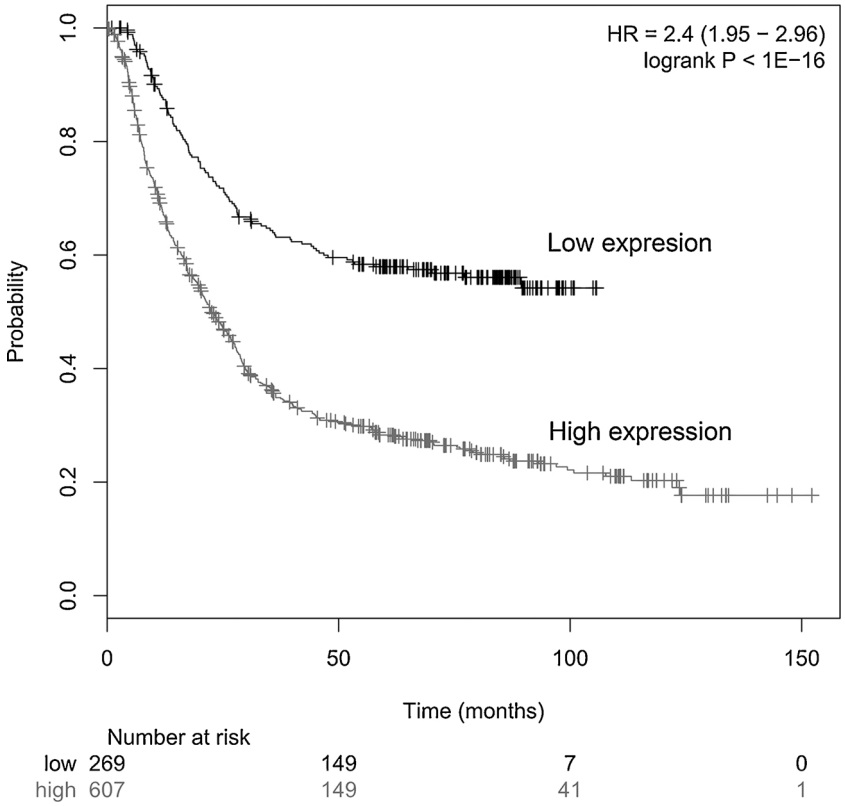

Fig. 2. Kaplan-Meier analysis of PKM2 level and survival rate of GC patients. Hazard ratio = 2.4, 95% confidence interval (1.95–2.96), log-rank p = 1E-16.

respectively, and MTT was added to each well after 24 h, 48 h, 72 h, or 96 h (Sigma-Aldrich, St. Louis, MO, USA). Absorbance at 570 nm was detected by an enzyme-labeled Minireader II (Bio-Rad, Hercules, CA, USA).

2.6. Soft agar colony formation

Agar clonogenic assay was utilized to evaluate tumor growth in vitro [16]. *PKM2* shRNA-expressing cells and mock cells were cultured in 60-mm dishes for 4 weeks respectively, and then, the quantity and size of colonies between these two kinds of GC cells were observed.

2.7. In vivo tumor growth

All animal studies were approved by the Ethics Committee on Animal Experimentation of Beijing Cancer Hospital. *PKM2* shRNA-expressing cells and mock cells were injected into BALB/c nude mice, respectively, which were observed for 3 weeks and then sacrificed. Size and weight of tumors from these two kinds of cells were measured and PKM2 levels were detected by IHC staining.

2.8. RT-PCR and real-time PCR

Total RNA was isolated utilizing an RNA isolation kit (Bioteke Corporation, Beijing, China), and used for reverse transcription according to an Easyscript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Primers were as follows: *PKM2* (forward: 5′-AGGCTGCCATCTACCACTTG-3′; reverse: 5′-GAAGATGCC ACGGTACAGGT-3′) and β-actin (forward: 5′-CGGGAAATCGTGCGTGA CATT-3′; reverse: 5′-CTAGAAGCATTTGCGGTGGAC-3′). Real-time PCR was performed using an RT-Cycler Real-Time PCR Detection System (CapitalBio Corp, Beijing, China) by the SYBR Green method. The primers were as follows: *PKM2* (same as for regular RT-PCR) and β-actin (forward: 5′-TTAGTTGCGTTACACCCTTTC-3′; reverse: 5′-ACCTTCACC GTTCCAGTTT-3′).

2.9. Western blotting

Western blotting was carried out as described previously [17]. Membranes were incubated with anti-p-AMPK, -AMPK, -p-AKT, -AKT, -BCL-2, -BAX, -PKM2, and -β-actin antibodies, followed by incubation with secondary antibodies.

2.10. Detection of apoptosis and reactive oxygen species (ROS)

Cell apoptosis was detected by flow cytometry following the instructions of the Annexin V-FITC apoptosis detection kit (Beijing Biosea Biotechnology Co. Ltd., Beijing, China). Intracellular ROS were detected using a ROS assay kit (Applygen, Beijing, China). Fluorescence was detected using flow cytometry (BD Biosciences, San Jose, CA, USA).

2.11. Measurement of ATP and lactate

ATP was detected with a firefly luciferase-based ATP Assay Kit (Beyotime, Haimen City, China), and lactate was detected with a Lactic Acid Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China).

2.12. Statistical analysis

SPSS13.0 software (SPSS, Chicago, IL, USA) was utilized for all of the statistical analyses. ($p < 0.05$) was considered as statistically significant.

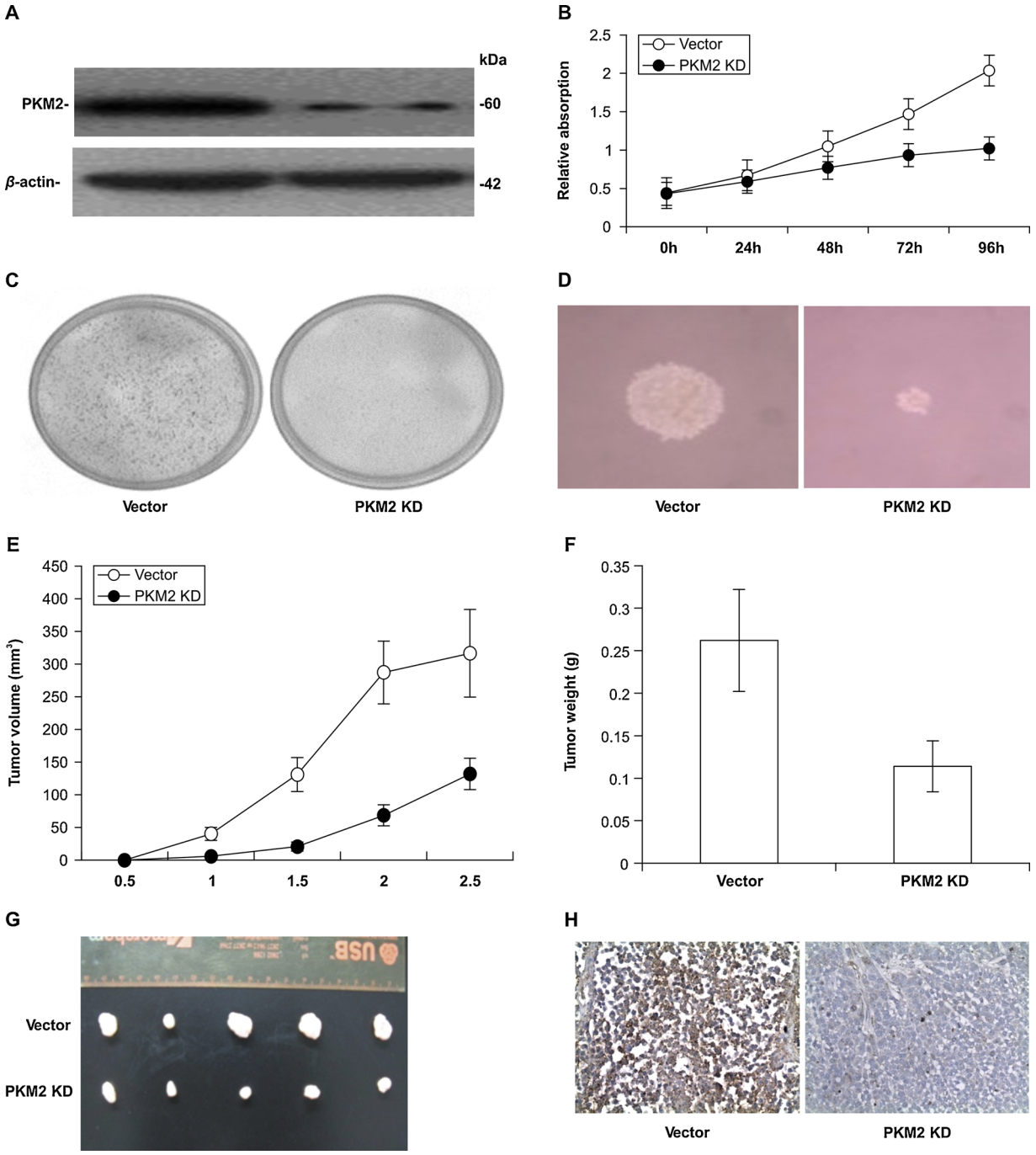

Fig. 3. Changes in cell growth following knockdown of *PKM2* by shRNA. (A) *PKM2* expression in *PKM2*-deficient cells compared with control cells. (B) Effects of *PKM2* knockdown on proliferation of gastric cancer cells as measured by MTT assay ($p < 0.05$). (C–D) Soft agar assays were used to measure colony-forming efficiency of *PKM2*-knockdown cells. (E–G) The size and weight of the tumors formed by *PKM2*-deficient cells compared with control cells ($p < 0.05$). (H) Immunohistochemical staining of tumors derived from *PKM2*-deficient cells and control cells.

3. Results

3.1. High expression of PKM2 is associated with poor prognosis in GC

In a previous study, we identified several genes in the glucometabolic pathway with altered expression of GC [17]. *PKM2* was found to be overexpressed in GC in comparison with adjacent and normal tissue specimens, and expression of PKM2 occurred in the GC tissue samples as well as the cell lines. In 6 GC cell lines, PKM2 was upregulated at both mRNA and the protein levels (Fig. 1A–B). Immuno-histochemical staining in tissue array confirmed that PKM2 levels were increased in GC specimens, 65% (34/52) of tumor tissues, and only 27% (10/37) of adjacent normal tissues (Fig. 1C, Table 1, $p < 0.001$). The KM-Plotter database was utilized to explore the relationship between PKM2 expression and clinical outcome. PKM2 upregulation was associated with poor survival of GC patients (Fig. 2, $p < 0.001$).

3.2. PKM2 promotes cell proliferation and tumorigenicity

The effect of *PKM2* knockdown on the behavior of GC cells was evaluated via MTT assays, soft agar assays, and tumor formation tests. Transfection with pSliencer3.0-PKM2 plasmid resulted in *PKM2*

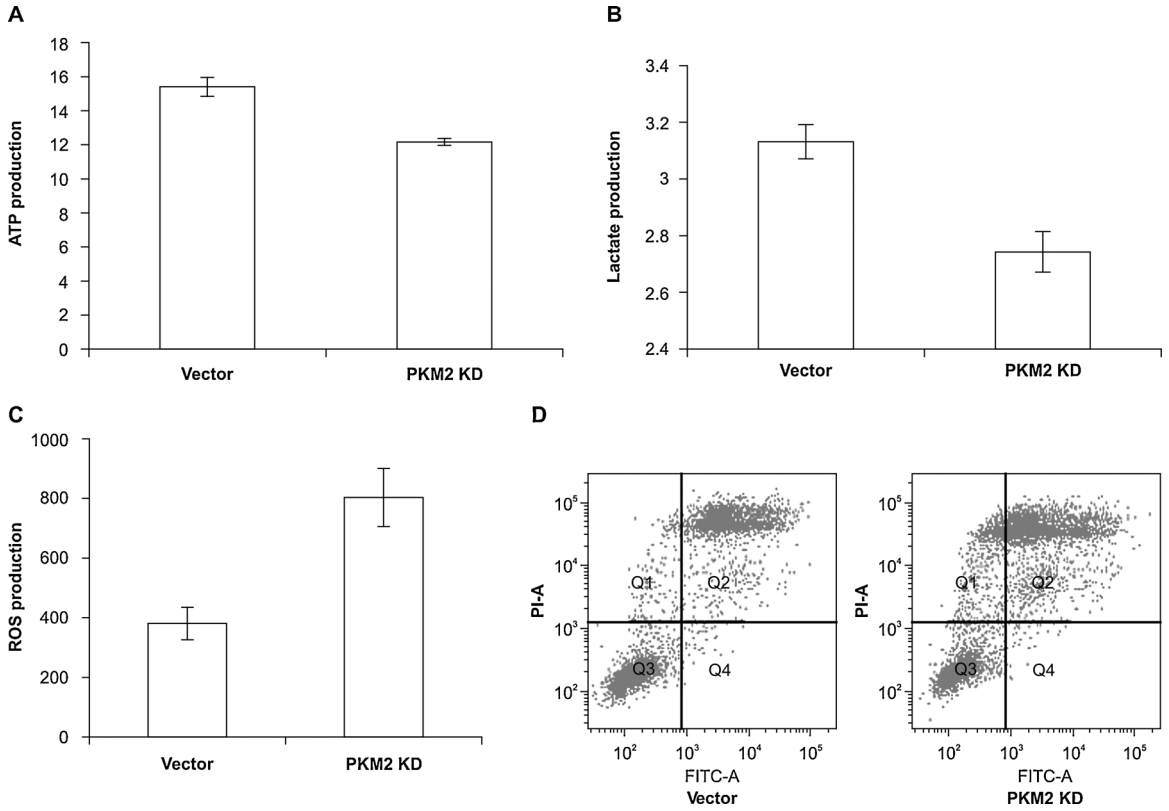

Fig. 4. Effects of *PKM2* knockdown by shRNA on glycolysis and apoptosis. (A) ATP, (B) lactate, and (C) ROS production in *PKM2*-deficient cells compared with control cells ($p < 0.05$). (D) Annexin V/PI staining was used to assess apoptosis of *PKM2*-deficient cells and control cells.

knockdown; these results are compared with control in Fig. 3 (Fig. 3A). MTT assays revealed that cell proliferation was suppressed in *PKM2*-deficient cells in comparison with mock cells (Fig. 3B, $p < 0.05$). *PKM2*-deficient cells developed smaller and fewer colonies compared with mock cells (Fig. 3C–D, $p < 0.05$). Moreover, tumors formed by *PKM2*-deficient cells in nude mice weighed less and were much smaller compared with those by mock cells (Fig. 3E–F, $p < 0.05$). Clonal origin of *PKM2*-deficient cells in the tumors was confirmed by IHC staining with an anti-PKM2 antibody (Fig. 3G–H).

3.3. PKM2 inhibits apoptosis

ATP and lactic acid levels were decreased in *PKM2*-deficient cells in comparison with mock cells ($p < 0.05$) as depicted in Fig. 4A–B. Moreover, ROS levels were higher in *PKM2*-deficient cells in comparison with mock cells (Fig. 4C, $p < 0.05$). Annexin V-FITC/PI labeling further showed that *PKM2* knockdown resulted in a higher ratio of apoptotic cells (Fig. 4D). These results suggested that *PKM2* knockdown suppressed glycolysis and promoted apoptosis of GC cells.

3.4. PKM2 inhibition activates AMPK and induces the mitochondrial apoptotic pathway

The effect of *PKM2* knockdown on various signaling pathways in GC cells was evaluated (Fig. 5). Phosphorylation of AMP-activated protein kinase (AMPK), a cellular energy sensor, was higher in *PKM2*-knockdown cells than in control cells, while total AMPK remained the same [18]. Additionally, the phosphorylation of AKT was decreased in *PKM2*-deficient cells in comparison with mock cells, which had a vital effect on tumor progression via proliferation stimulation and apoptosis inhibition, while total AKT did not change [19]. Within the apoptotic pathway, BAX expression was higher, while BCL-2 expression was lower in *PKM2*-knockdown cells than in mock cells, indicating the activation of ROS-induced apoptosis.

4. Discussion

We found that increased PKM2 expression was associated with poor survival of GC patients. In addition, downregulation of PKM2 inhibited glucose metabolism and proliferation, and induced apoptosis of GC cells. Furthermore, *PKM2* knockdown activated AMPK and induced the mitochondrial apoptotic pathway, suggesting that PKM2 is a novel target for the treatment of GC.

The essential difference between cancer cells and normal cells is the aberrant proliferative capacity of cancer cells, as cancer cells have an excessive need for nutrients and energy [3]. In order to sustain this high demand, cancer cells have developed altered metabolic pathways [7]. In the 1920s, Warburg first proposed that when an ample supply of oxygen is present, cancer cells prefer glycolysis to consume glucose, whereas normal cells depend on oxidative phosphorylation; this phenomenon is termed the “Warburg effect” [4,5]. Thus, enzymes involved in the glycolytic pathway may be regulated in cancers and may exert a critical effect on cancer cell growth and tumorigenesis [8]. Consistent with this, we found that *PKM2* was greatly upregulated in GC tissues and cell lines, indicating that PKM2 has an essential effect on the biological behavior of GC.

This study also found that *PKM2* knockdown in GC cells suppressed glycolysis, resulting in a reduction in ATP and lactate. Supporting this effect, the low energy status of the cells activated AMPK, a sensor of cellular energy. AMPK, a heterotrimer with one catalytic subunit (α) and two regulatory subunits (β and γ), serves as a highly conserved serine/threonine protein kinase. An increase in AMP/ATP ratio results in AMPK activation, which indicates that energy homeostasis is compromised [18]. AMPK activation occurs under certain conditions such as oxidative stress, hypoxia, and nutrient deprivation, which depletes cellular ATP and elevates AMP levels [20]. Recent studies showed that

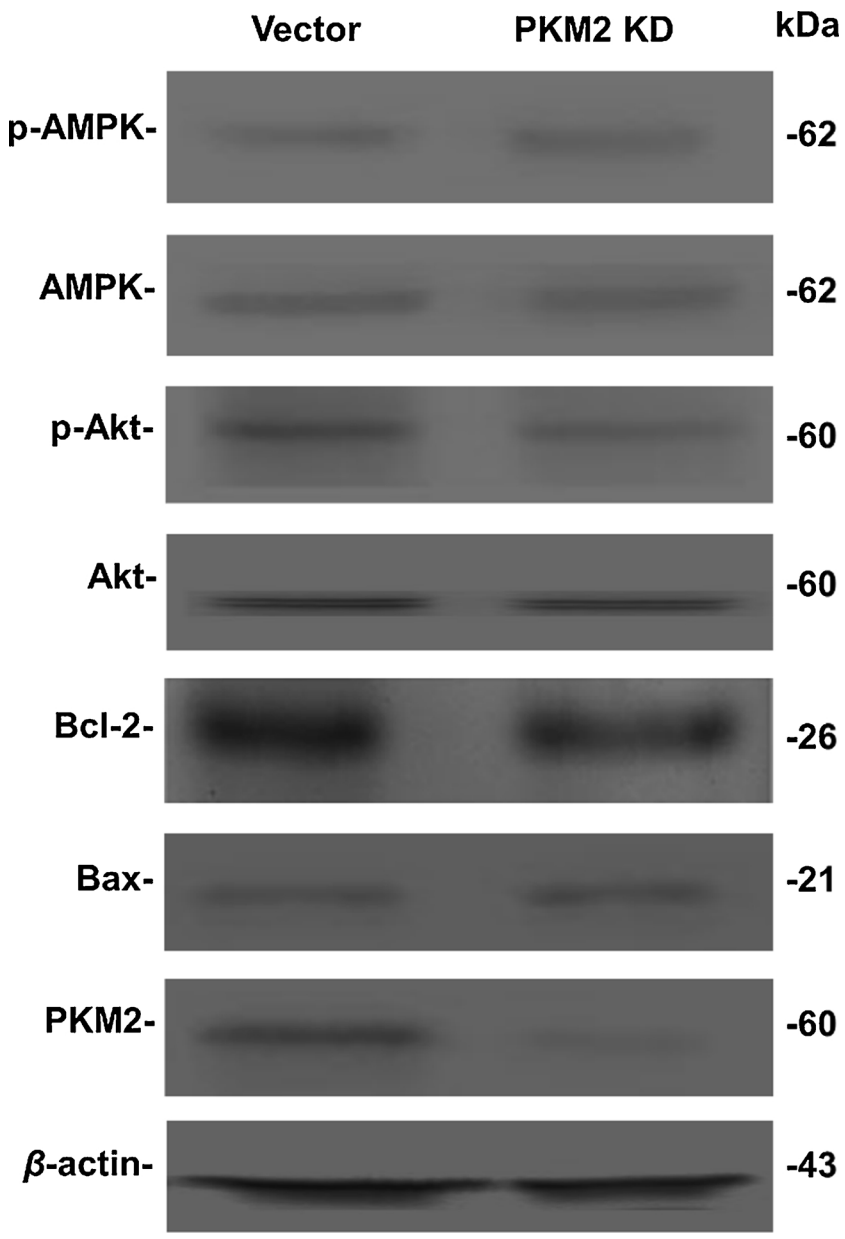

Fig. 5. Molecular mechanism underlying the effect of *PKM2* knockdown on gastric cancer cells. The phosphorylation of AMPK was increased, while that of AKT was decreased; BAX expression was increased, while BCL-2 expression was decreased.

AMPK inactivation coordinates with oncogenes to promote cancer progression [21]. AMPK activation had an adverse effect on AKT-dependent pathways, which are crucial in carcinogenesis [22]. Consistent with these previous studies, data from this study demonstrated that *PKM2* knockdown suppressed cell growth and increased phosphorylation (i.e., activation) of AMPK, concurrently with reduced AKT phosphorylation. These data support the role of PKM2 in mediating essential tumor-associated pathways.

Conversely, glucose metabolism serves as the nexus between cell growth and apoptosis [23]. Metabolic stress such as ROS can trigger the mitochondria-dependent apoptotic pathway by inducing mitochondrial permeabilization and the release of cytochrome c [24]. Moreover, both the pro-apoptotic (e.g., BAX) and anti-apoptotic (e.g., BCL-2) members of the BCL-2 superfamily are involved in mediating the mitochondria-dependent apoptotic pathway [25,26]. In support of PKM2 as a modulator of GC cell apoptosis, we found that *PKM2* knockdown enhanced ROS production and promoted mitochondria-dependent apoptosis through downregulation of BCL-2 and upregulation of BAX.

In conclusion, PKM2 is crucial in the regulation of proliferation, apoptosis, and glycometabolism within GC cells. Results of this study demonstrated that inhibition of PKM2 represents a novel chemotherapeutic strategy for the treatment of gastric cancer.

Conflict of interests

The authors declare no competing interests.

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References

[1] E. Van Cutsem, X. Sagaert, B. Topal, K. Haustermans, H. Prenen, Gastric cancer,

Lancet 388 (2016) 2654–2664.
[2] Global Burden of Disease Cancer Collaboration, Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: A systematic analysis for the global burden of disease study, JAMA Oncol. 3 (2017) 524–548.
[3] O. Warburg, On the origin of cancer cells, Science 123 (1956) 309–314.
[4] O. Warburg, Über den stoffwechsel der carcinomzelle, Naturwissenschaften 12 (1924) 1131–1137.
[5] M.G. Vander Heiden, R.J. DeBerardinis, Understanding the intersections between metabolism and cancer biology, Cell 168 (2017) 657–669.
[6] N.N. Pavlova, C.B. Thompson, The emerging hallmarks of cancer metabolism, Cell Metab. 23 (2016) 27–47.
[7] L.K. Boroughs, R.J. DeBerardinis, Metabolic pathways promoting cancer cell survival and growth, Nat. Cell Biol. 17 (2015) 351–359.
[8] T.N. Seyfried, R.E. Flores, A.M. Poff, D.P. D'Agostino, Cancer as a metabolic disease: implications for novel therapeutics, Carcinogenesis 35 (2014) 515–527.
[9] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[10] K. Imamura, T. Tanaka, Pyruvate kinase isozymes from rat, in: W.A. Wood (Ed.), Methods in Enzymology, 90 Academic Press, 1982, pp. 150–165.
[11] T.L. Dayton, T. Jacks, M.G. Vander Heiden, PKM2, cancer metabolism, and the road ahead, EMBO Rep. 17 (2016) 1721–1730.
[12] W.J. Israelsen, M.G. Vander Heiden, Pyruvate kinase: function, regulation and role in cancer, Semin. Cell Dev. Biol. 43 (2015) 43–51.
[13] V.M. Liu, M.G. Vander Heiden, The role of pyruvate kinase m2 in cancer metabolism, Brain Pathol. 25 (2015) 781–783.
[14] Z. Li, P. Yang, Z. Li, The multifaceted regulation and functions of PKM2 in tumor progression, Biochim. Biophys. Acta Rev. Cancer 1846 (2014) 285–296.
[15] A.M. Szász, A. Lánczky, Á. Nagy, S. Förster, K. Hark, J.E. Green, A. Boussioutas, R. Busuttil, A. Szabó, B. Győrffy, Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients, Oncotarget 7 (2016) 49322–49333.
[16] Y. Kajiwara, S. Panchabhai, V.A. Levin, A new preclinical 3-dimensional agarose colony formation assay, Technol. Cancer Res. Treat. 7 (2008) 329–334.
[17] H. Li, J. Wang, H. Xu, R. Xing, Y. Pan, W. Li, J. Cui, H. Zhang, Y. Lu, Decreased fructose-1,6-bisphosphatase-2 expression promotes glycolysis and growth in gastric cancer cells, Mol. Cancer 12 (2013) 110.
[18] D.G. Hardie, B.E. Schaffer, A. Brunet, AMPK: an energy-sensing pathway with multiple inputs and outputs, Trends Cell Biol. 26 (2016) 190–201.
[19] B.D. Manning, L.C. Cantley, AKT/PKB signaling: navigating downstream, Cell 129 (2007) 1261–1274.
[20] D.G. Hardie, AMPK: positive and negative regulation, and its role in whole-body energy homeostasis, Curr. Opin. Cell Biol. 33 (2015) 1–7.
[21] B. Faubert, E.E. Vincent, M.C. Poffenberger, R.G. Jones, The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator, Cancer Lett. 356 (2015) 165–170.
[22] J.Y. Liang, G.B. Mills, AMPK: A contextual oncogene or tumor suppressor? Cancer Res. 73 (2013) 2929–2935.
[23] J.L. Andersen, S. Kornbluth, The tangled circuitry of metabolism and apoptosis, Mol. Cell 49 (2013) 399–410.
[24] E.F. Mason, J.C. Rathmell, Cell metabolism: an essential link between cell growth and apoptosis, BBA Mol. Cell Res 1813 (2011) 645–654.
[25] T. Moldoveanu, A.V. Follis, R.W. Kriwacki, D.R. Green, Many players in BCL-2 family affairs, Trends Biochem. Sci. 39 (2014) 101–111.
[26] P.E. Czabotar, G. Lessene, A. Strasser, J.M. Adams, Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy, Nat. Rev. Mol. Cell Biol. 15 (2014) 49–63.